of binding to a B cell linker protein, and for screening for a bioactive agent capable of modulating the bioactivity of a B cell Linker Protein.

Amendments:

Claims 1-22 have been canceled without prejudice or disclaimer. New claims 23-34 have been added.

Response to rejections:

35 U.S.C. § 101:

Claim 16 stands rejected under 35 U.S.C. § 101 as not being supported by either a specific asserted utility or a well established utility. In this regard, the Office Action states at page 2: " the specification fails to identify any disease or condition treatable with a BLNK protein in a pharmaceutical composition." Applicants respectfully traverse the rejection.

The new guidelines for determining utility

To review the proper standard for supporting rejections based on lack of utility under § 101 (or the how to use requirement of § 112, first paragraph), Applicant turns to the new guidelines set forth by the Patent Office for determining utility, referred to herein as "the guidelines". As the guidelines were issued by the Patent Office, Applicant has not enclosed a copy herein. First, Applicant points out that these guidelines are applicable to rejections both for lack of utility under 35 U.S.C. § 101 and for failing to teach "how to use" for *in vivo* therapeutic or pharmacological utility under 35 U.S.C. § 112, first paragraph (page 296, left column, lines 30-35 and page 300, right column of the guidelines).

The guidelines specifically indicate that a rejection is proper **only** in the "rare instance" where an assertion of specific utility for the invention made by an applicant is not credible to one of ordinary skill in the art (pages 296-298 of the guidelines).

Regarding the qualifications for "credibility", the guidelines indicate that a specific assertion of utility not only creates a presumption of utility, but also is deemed credible

"unless (a) the logic underlying the assertion is seriously flawed, or (b) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion" (page 303 of the guidelines, emphasis supplied).

Applicant also points out the discussion in the guidelines with regard to *in vitro* experimental data and the requirement for human clinical data. Specifically, page 306, right column, states:

> If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays....almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process. In no case has a Federal court required an applicant to support an asserted utility with data from human clinical trials. (Emphasis supplied).

This section of the guidelines further states that primary responsibility for determining the existence of a therapeutic or pharmacological utility for a compound, composition or method in humans lies with those who are "especially skilled in the art" at the U.S. Food and Drug Administration, not with the U.S. Patent Office.

Summary of the guidelines

Applicant, therefore, summarizes the new guidelines to state the following:

(a) all that is needed to create a presumption of utility is a credible assertion of utility made by the Applicant;

(b) the assertion is considered credible unless the underlying logic of the assertion is seriously flawed or the logic is valid but the facts are not consistent with the logic; and

(c) *in vitro* data is invariably sufficient to establish therapeutic or pharmacological utility for a compound so long as a reasonable correlation between the data and the assertions of utility can be established.

Under the proper standard, the invention has utility

Applicant submits that (a) an assertion of utility has been made, (b) the logic is not seriously flawed and the facts are consistent with the logic, and (c) that the data presented in the specification is reasonably correlated with the purported utility.

The assertion of utility

Applicant asserts that a pharmaceutical composition comprising a BLNK protein

Not Specific

can be used to treat diseases involving B cells. Particularly, Applicant asserts that a pharmaceutical compound comprising a BLNK protein is useful for the treatment of diseases associated with altered B cell activation, including uncontrolled B cell growth, perturbed B cell differentiation, and perturbed immune system function.

The logic is not seriously flawed

The logic underlying the assertion of utility is that if a BLNK protein affects signaling mechanisms that lead to B cell activation, and alterations in B cell activation are associated with particular dysfunctions, then said dysfunctions will be beneficially treated by administration of a pharmaceutical compound comprising a BLNK protein. Put in an even more simplified manner, the premise is that administration of a substance A which affects mechanisms regulating the presence of process B, will beneficially treat dysfunctions associated with the inappropriate presence or absence of process C. Applicant submits that the determination of utility at this point in the analysis does not require a review of previous studies on this subject, nor a review of the contents of the specification. Applicant merely submits that this underlying logic is "not seriously flawed". Therefore, under the new guidelines, the assertion of utility made by the Applicant is thus far presumed valid.

The facts are consistent with the logic

Moving on to an examination of the facts, Applicant submits that the facts presented are consistent with the logic underlying the assertion of utility. Particularly, the facts show that BLNK protein affects signaling mechanisms that lead to B cell activation. More specifically, the facts show that BLNK protein interacts with intracellular signal transducing proteins that transduce signals leading to B cell activation, and that BLNK protein is capable of modulating at least one intracellular signal that leads to B cell activation. The facts further show that these protein associations take place when signals inducing B cell activation are initiated, and that BLNK protein normally undergoes post-translational modification following signal initiation. These data suggest that BLNK protein normally plays a role in B cell activation.

The facts also show that aberrant B cell activation is associated with particular

dysfunctions, including uncontrolled B cell growth, perturbed B cell differentiation, and perturbed immune system function. More recent studies support Applicant's original assertion of utility, further showing that changes in signaling mechanisms leading to B cell activation and associated with BLNK protein are associated with such dysfunctions, and that changes in BLNK proteins themselves are associated with such dysfunctions.

Applicant submits that the facts presented show that BLNK proteins are capable of affecting signaling mechanisms that lead to B cell activation, and that altered B cell activation is associated with dysfunctions. Moreover, the facts show that the signaling mechanisms leading to B cell activation are also associated with dysfunction, and more particularly, that altered BLNK protein is associated with dysfunction. As such, Applicant submits that a pharmaceutical compound comprising a BLNK protein has potential utility for the treatment of dysfunctions associated with B cell activation.

Molecular interactions of BLNK protein and signaling in B cell activation

The identified human B cell Linker Protein 1 (BLNK1 protein) is an intracellular phosphoprotein specifically expressed in B-cells which is immediately phosphorylated on five tyrosine residues following B cell receptor (BCR) activation. This phosphorylation is effected by the BCR-responsive protein tyrosine kinase Syk, and the temporal nature of the phosphorylation events suggests BLNK1 proteins may participate in signal transduction leading to B cell activation.

BLNK1 associates with two proteins that effect signals known to be required for BCR-mediated B cell activation, namely Grb2 and PLC-γ. BLNK1 protein interacts with Grb2, which effects ras signaling downstream of the BCR that is essential for BCR-mediate B cell activation. Further, alterations in ras signaling are frequently associated with abnormal growth. BLNK1 also interacts with PLC-γ, which effects calcium signaling downstream of the BCR that is also essential for BCR-mediated B cell activation.

Further, BLNK1 has been shown to modulate calcium flux following BCR activation; overexpression of wildtype BLNK1 protein leads to enhanced signaling following BCR activation, while forced expression of a phosphorylation mutant BLNK

protein decreases calcium signaling following BCR activation. In addition, preliminary experiments refered to in the present specification (data not shown) indicate that BLNK1 may effect cytoskeletal changes brought about by BCR activation through associations with the adaptor protein nck and the proto-oncogene product vav following BCR activation.

In summary, BLNK protein:

i) is immediately phosphorylated on tyrosine following the initiation of a signal that will lead to B cell activation; and

ii) physically associates with signal transducing proteins effecting B cell activation during B cell activation; and

iii) directly effects calcium flux, a critical signal for B cell activation following BCR stimulation; and

iv) physically associates with the product of the proto-oncogene vav during B cell activation; and

v) is implicated as an effector of cytoskeletal rearrangements during B cell activation (cytoskeletal modulation being involved in tumor growth and migration)

Applicant also points out that the concept of altered signal transduction effecting aberrant cell differentiation, migration, and proliferation was common at the time the present application was filed.

Altered B cell activation is associated with dysfunctions

At the time the present application was filed, it was well known that B cell hypoactivation (including the absence of mature B cells) was associated with immunodeficiency disorders while B cell hyperactivation was associated with autoimmune disorders (Roitt, Essential Immunology, pp. 35-36; Rosen et al., Clin Exp Immunol, 109:S1-S28, 1997, Exhibit A, enclosed). It was also well known that certain B cell tumors were associated with alterations in B cell activation (eg. Aguilar-Santileses et al., Leukemia 8:1146-1152, 1994, Exhibit B, enclosed).

Findings subsequent to the filing of the present application support Applicant's assertion that pharmaceutical compounds comprising BLNK proteins are useful for the

treatment of diseases involving B cells. As the signaling mechanisms regulating B cell activation have been studied in pathological contexts, they have been shown to be associated with particular diseases involving B cells. Alterations in calcium signaling have been shown to occur in B cell leukemia, and responses to BCR stimulation are correlated with clinical disease progression in chronic lymphocytic leukemia (Meinhardt et al., J. Mol. Med. 77:282-293,1999, Exhibit C, enclosed). Moreover, two reports in the journal *Science* disclose that BLNK protein function is essential for B cell function in mice and humans. Pappu *et al.* (Science, **286**:1949-1954, 1999, Exhibit D, enclosed) disclose that disruption of the *BLNK1* locus in mice blocks the formation of precursor B cells from progenitor B cells, leading to the near complete absence of mature B cells. Minegishi *et al.* (Science, **286**:1954-1957, 1999, Exhibit E, enclosed) disclose that a naturally occurring mutation in the human *BLNK1* locus also results in the absence of mature B cells and precursor B cells, despite the presence of progenitor B cells. This is similar to the developmental block seen in X-linked aggamglobulonemia (LeBien, Blood 96:9-23, 2000 Exhibit F, enclosed). Additionally, *BLNK1* has been shown to be essential for BCR signaling and BCR-mediated B cell activation (Xu *et al.*, Int. Immunol. **12**:397-404, 2000, Exhibit G, enclosed; Hayashi *et al.*, PNAS, **97**:2755-2760, 2000, Exhibit H, enclosed; Ishiai *et al.*, Immunity, **10**:117-125, 1999, Exhibit I, enclosed).

It appears to be the Examiner's position that regardless of whether Applicant has shown that BLNK activity modulates signal transduction leading to B cell activation, such an effect does not necessarily indicate that BLNK protein will have a therapeutic effect on diseases associated with altered B cell activation. Again, Applicant submits that claims 21 and 22 satisfy the requirements of §112, first paragraph, and respectfully request removal of the rejection and allowance of the claims.

A specification that discloses information on how to make and use the invention must be accepted unless the Patent Office provides sufficient reason to doubt the accuracy of the disclosure. If the Patent Office does present doubt to the accuracy of the disclosure, then such a rejection can be overcome by suitable proofs such as subsequent data confirming the assertions made in the application. In re Marzocchi, 439 F.2d 220, 169

U.S.P.Q. 367 (C.C.P.A. 1971). In this case, assuming *arguendo* that the Patent Office raised a doubt to the accuracy of the assertions made in the specification, Applicant submits that this rejection has been overcome by the data presented in the present application which confirms the assertions made in the present application.

The data is reasonably correlated with the purported utility

Applicants submit that there exists a specific asserted utility for the compound of Claim 16 and that the claimed invention satisfies the requirements of U.S.C. § 101. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of Claim 16.

Claims 21 and 22 stand rejected under 35 U.S.C. §101 as not being supported by either a specific asserted utility or a well established utility. In this regard, the Office Action states at page 4: "...the asserted potential treatment utility [of bioactive agents capable of binding to BLNK protein] for B cell lymphomas and autoimmune diseases which have hyperactivated B cells (pages 19-20) is not supported by the disclosure." Applicants respectfully traverse the rejection.

As discussed above, the new guidelines set forth by the Patent Office in regard to utility may be summarized as follows:

(a) all that is needed to create a presumption of utility is a credible assertion of utility made by the Applicant;

(b) the assertion is considered credible unless the underlying logic of the assertion is seriously flawed or the logic is valid but the facts are not consistent with the logic; and

(c) *in vitro* data is invariably sufficient to establish therapeutic or pharmacological utility for a compound so long as a reasonable correlation between the data and the assertions of utility can be established.

Under the proper standard, the invention has utility

Applicant submits that (a) an assertion of utility has been made, (b) the logic is not seriously flawed and the facts are consistent with the logic, and (c) that the data presented in the specification is reasonably correlated with the purported utility.

Assertion of Utility

Applicant respectfully points out that claims 21 and 22 are not drawn to the use of bioactive agents for the treatment of disease. Rather, the claims are directed to methods of screening agents for their ability to bind BLNK proteins and/or modulate BLNK protein activity. These agents need not have therapeutic utility, but must meet the requirement s of §101 and §112.

Certainly, as those in the art will appreciate, these screening methods find utility in a variety of ways including use in the elucidation of the mechanism, activity, or function of BLNK proteins, as well as in the study of signal transduction mechanisms and B cell activation directly. The tissue localization, molecular interactions, response to BCR activation, and involvement in calcium signaling make BLNK proteins attractive targets for manipulation in the study of B cell activation and associated dysfunctions. Further, claims to methods of screening for bioactive agents that bind proteins of interest are most frequently found allowable, without a prerequisite assertion of therapeutic utility before the fact.

Finally, recent studies elucidating the function of BLNK proteins (Pappu et al, Exhibit D; Minegishi et al, Exhibit E; described above and enclosed) have heightened the desire for bioactive agents that affect BLNK protein activity.

Accordingly, Applicant submits that claims 21 and 22 meet the requirements for utility under §101 and §112.

The logic is not seriously flawed

The logic of the assertion is that if a bioactive agent is capable of binding to a BLNK protein, said bioactive agent may affect some property of a BLNK protein, as would be appreciated by one of reasonable skill in the art. Obviously a bioactive agent capable of modulating the activity of a BLNK protein may alter some property of a BLNK protein. By affecting some property of a BLNK protein, an agent is potentially useful for the study of BLNK proteins themselves, as well as the physiological systems that BLNK proteins can affect.

The facts are consistent with the logic

The facts are consistent with the logic underlying the asserted utility of screening methods for identifying agents capable of binding to BLNK proteins and/or modulating their activity. The claims call for bioactive agents capable of binding to BLNK proteins and/or modulating their activity. For argument, this property must be ascribed to the as yet unidentified agents. It is obvious that a bioactive agent capable of modulating BLNK protein activity could affect some property of a BLNK protein. Further, it would be obvious to one of reasonable skill in the art that a bioactive agent capable of binding to a BLNK protein could affect some property of a BLNK protein. Accordingly, the logic underlying the assertion of utility of the screening methods is consistent with the facts. Moreover, Applicant reiterates that claims to methods of screening for bioactive agents that bind proteins of interest are most frequently found allowable, without a prerequisite assertion of therapeutic utility before the fact.

Applicants submit that Claims 21 and 22 satisfy the requirements of U.S.C. § 101 and §112. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of claims 21 and 22.

Rejections under §112, first paragraph

Claims 1-3, 7-14,16-22 stand rejected under 35 U.S.C. §112, first paragraph. Applicants respectfully traverse.

The Examiner states that: "The claims are drawn to any recombinant nucleic acid encoding any BLNK protein and to any BLNK protein, ie., any "molecules which interact with either (sic) Grb2, PLCγ or SYK" as stated in the last four lines at page 2 of the specification." and rejects the claims as being too broad. The Examiner's main point seems to be that the definition of "BLNK protein", as used in the claims, is too broad. Accordingly, Applicant has amended the pending claims to direct them to specific SEQ ID NOs. With particular regard to claims 3, 4 and 7, the Applicant notes that nucleic acids hybridizing to SEQ ID NO:2 under high stringency conditions were found enabled as evidenced by claim 2.

Rejections under 102(b) and 102 (e): Anticipation; Rejections under 103(a): Obviousness

Claims 1, 8, 11 and 17-20 stand rejected under 35 U.S.C. §102(b) as being anticipated by Nagai et al. Claims 1, 2, 8, 10, 11 and 17-20 stand rejected under 35 U.S.C. §102(b) as being anticipated by Jackman et al. Claims 1, 8, 10, 11, and 17-20 stand rejected under 35 U.S.C. §102(b) as being anticipated by Richard et al. Claim 21 stands rejected under 35 U.S.C. §102(e) as being anticipated by Morris et al. Claims 1, 2, and 6-8 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Nagai et al, Jackman et al. or Richard et al. in view of Kaufman.

The rejections over the prior art depend from an interpretation of "BLNK protein" as stated in the Office Action at page 9: "..the claimed "BLNK protein" is interpreted as "molecules which interact with either (sic) Grb2, PLC-γ or SYK" as stated in the last four lines at page 2 of the specification."

Applicants submit that the claims as amended recite SEQ ID NOs 1 and 2, and therefore the rejections should be withdrawn.

Rejections based on statutory double patenting

Claims 4-6 stand rejected under 35 U.S.C. 101 as claiming the same invention as that of claims 1-3 of prior U.S. Patent No. 5,994,522.

Applicants submit that the claims as amended are patentably distinct from claims 1-3 of U.S. Patent No. 5,994,522.

Rejections based on obviousness-type double patenting

Claims 7-9 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 5-7 of U.S. Patent No. 5,994,522.

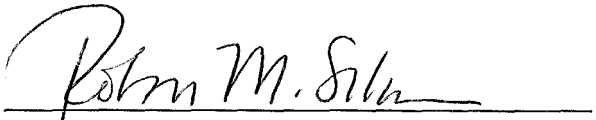
The Applicant requests that this rejection be held in abeyance until otherwise allowable subject matter is found.

CONCLUSION

Applicants respectfully submit that the claims are in condition for allowance. Early notice to that affect is solicited. If a telephone conference would expedite the prosecution of this patent application, the Examiner is invited to call the undersigned attorney at (415) 781-1989.

Respectfully submitted,

FLEHR HOHBACH TEST
ALBRITTON & HERBERT LLP



Robin M. Silva
Reg. No. 38,304

Four Embarcadero Center, Suite 3400
San Francisco, CA 94111-4187
Telephone: (415) 781-1989

Dated:

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Primary Immunodeficiency Diseases

Report of a WHO Scientific Group

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Primary Immunodeficiency Diseases

REPORT OF A WHO SCIENTIFIC GROUP*

1 INTRODUCTION

The initial barriers to infection are skin, mucous membranes and the substances they secrete. When infectious agents penetrate these barriers other non-specific host factors such as cytokines and complement come into play. These components together with the specific immune mechanisms of antibodies and lymphocytes constitute the immune system. The complex of interacting factors and cells provides the initial innate non-specific defence and subsequently the acquired specific defence mechanisms for resistance to infection.

The Primary Immunodeficiency Diseases result from innate defects of the immune system. As a consequence, recurrent protozoal, bacterial, fungal and viral infections of varying severity ensue. The immune system can also be adversely affected secondarily by a variety of pathological conditions (including malignancy, metabolic diseases and malnutrition) and drugs; these result in Secondary Immunodeficiencies.

Both Primary and Secondary Immunodeficiencies result in a similar spectrum of illness – recurrent or persistent infections. Since the relationship between immunity and infection is interactive, infection may cause as well as result from immunodeficiency. Many infectious agents, including the human immunodeficiency virus (HIV), have both specific and non-specific effects on the immune system.

Study of patients with Primary Immunodeficiency Diseases has expanded our understanding of immunity. Recent progress in immunobiology and genetics has, with increasing precision, identified the causes of many of the Primary Immunodeficiency Diseases; diagnosis and therapy can as a result be more specific and effective.

2 CELLULAR BASIS OF THE IMMUNE RESPONSE

The progenitors of T cells, B cells and natural killer (NK) cells are derived from the same multipotent haematopoietic stem cells (HSC) that give rise to other types of blood cells. Cells of the monocyte-macrophage series, including Langerhans cells and dendritic cells, process and present antigen to the T and B cells both early in their development and later after they reach maturity (see Fig. 1).

Progenitor cells migrate from the circulation into the epithelial thymus where they interact with the stromal cells and their soluble products to undergo cell division, clonal selection and maturation. The T-lineage cells interact with their microenvironment through cell surface glycoproteins that serve as adhesion molecules and receptors coupled to signal transduction elements. An early thymocyte decision determines the choice of one of two pathways of differentiation. Progenitor cells (pro-T cells) may rearrange and express γδ T cell receptor (TCR) genes together with the CD3 complex of proteins to become γδ T cells. Alternatively, precursor cells may rearrange their VDJβ genes and express the completed β chain together with a pre-T α chain (pTα) and the CD3 protein signalling complex. These pre-T cells then rearrange their VJα genes to produce α chains and become αβ T cells. Cells of this lineage (immature T cells) initially express both CD4 and CD8 molecules that interact respectively with MHC class II or class I molecules on thymic stromal cells to influence their maturation into $CD4^+$ or $CD8^+$ T cells. Positive or negative selection of immature αβ T-cell clones is determined by the affinity of the TCR interaction with self antigens presented as peptide fragments within the grooves of MHC class II and/or class I molecules on thymic stromal cells. The γδ T cells do not express CD4 or CD8 molecules during their intrathymic maturation, and intrathymic clonal selection is probably not essential for their development. The γδ T cells can be subdivided on the basis of their utilization of either the γ1 or γ2 constant region genes together with preferred sets of VDJδ genes.

T-cell development in the thymus requires integrity of each of the TCR/CD3 components, CD4, CD8 certain cytokines, cytokine receptors, and their signal transduction partners. Later, when they migrate to the periphery, T cells may undergo selective clonal activation leading to proliferation and maturation. Antigen activation involves the interaction of T-cell receptors with antigen fragments held within the grooves of MHC class I or class II molecules. The activated αβ T cells begin to produce lymphokines like IL-2 and to express high affinity receptors for this lymphokine. The interaction of IL-2 with its receptor modulates T-cell growth and effector function.

The role of γδ T cells is presently unclear, but their acquisition of CD8 in peripheral tissues may enhance interaction with target cells bearing class I (or class I-like) MHC gene products. There is increasing evidence that γδ cells require exogenous growth factors, such as IL-7, produced by αβ T cells or other cell types. Crosstalk between αβ and γδ T cells may co-ordinate their activities to control immune responses.

The development of B lineage cells is a multifocal process which is concentrated in fetal liver before bone marrow becomes the major haematopoietic organ. Progenitor cells (pro-B cells) receiving signals from local stromal cells begin to divide, rearrange their immunoglobulin VDJ gene segments, and give rise to clones of pre-B cells. The pre-B cells express low levels of receptors composed of μ heavy chains, a surrogate light chain complex of V pre-B and λ-5 proteins, and an Igα/Igβ dimer. The cytoplasmic tails of the Igα and Igβ chains contain immunoregulatory tyrosine activation motifs (ITAMs) that are needed for signal transduction. Pre-B cells thus equipped may then rearrange VJ gene segments in their light chain loci to become immature IgM^+ B cells. Immature B cells are easily tolerized or killed by premature stimulation via their antigen receptors. After migrating from the bone marrow, the B cells mature, express IgD

*SIGNATORIES
Fred S. Rosen, Chairman, Ralph J.P. Wedgwood, rapporteur, USA; Martha Eibl, Austria; Alain Fischer, France; Fernando Aiuti, Luigi Notarangelo, Italy; Tadamitsu Kishimoto, Japan; Igor D. Resnick, Russia; Lennart Hammarstrom, Sweden; Reinhard Seger, Switzerland; Helen Chapel, Ronald A. Thompson, UK; Max D. Cooper, Ralf S. Geha, Robert A. Good, Thomas A. Waldmann, USA. Report of a meeting held on 17 October 1996 at the Jenner Museum, Berkeley, Glos., UK.

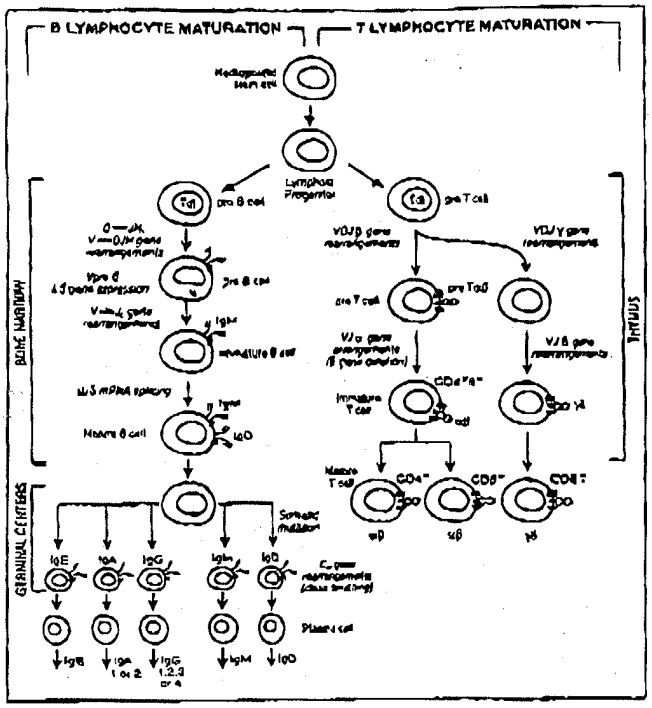

Fig. 1. Development of T and B lymphocytes.

antigen receptors, and respond to antigens and CD4+ T-cell help by undergoing proliferation and plasma cell differentiation.

In germinal centres, B cells see antigen on follicular dendritic cells and interact with helper T cells to undergo proliferation, somatic mutation and Ig class switching. Germinal centre B cells that produce antibodies of relatively high antigen affinity are selected to give rise to plasma cells that produce different Ig isotypes (IgM, IgG, IgA or IgE) or become recirculating memory B lymphocytes. Cell interaction molecules important for the germinal centre response include complement receptors and CD40 on B cells (and dendritic cells) and the CD40 ligand on activated T cells. The activated B cells express CD80 and CD86 molecules that in turn interact with CD28 and CTLA molecules on T cells to modulate their response. Cytokines (IFNγ, IL-2, 4, 5, 6, 7, 10, and 12 to 15) and their cell surface receptors are also important in facilitating the genetically restricted interactions between antigen presenting cells and T cells to elicit cell-mediated immunity, and the CD4+ T-cell interaction with B cells required for humoral immunity. CD4+ helper T cells (Th) are directed by different cytokines along two functionally-distinct pathways, Th1 and Th2. Th1 helper T cells characteristically produce cytokines that enhance inflammation, IL-2, IFNγ and TNF, whereas Th2 helper T cells produce cytokines that enhance antibody production, namely IL-4, IL-5, IL-10 and IL-13. IL-12 and IFNγ facilitate the Th1 cell differentiation pathway involved in cell-mediated inflammatory responses. Conversely, IL-4 facilitates the Th2 pathway of cell differentiation to yield helper T cells that promote the antibody response. In parallel with the CD4 cells, there are two groups of CD8 cytotoxic T cells, Tc1 and Tc2, each of which produces distinct sets of cytokines.

The basic cellular elements of the immune system are well established by 15 weeks of human gestation. Nevertheless, the system is functionally immature at birth and requires antigen selection and experience to achieve full maturation during infancy.

3 GENETIC BASIS OF THE IMMUNE RESPONSE

Immunoglobulins, which are tetramers of two heavy and two light chains, serve as antigen receptors on B cells, and the secreted antibodies are the effectors of the humoral immune system. Heavy chains of immunoglobulins are encoded by genes on chromosome 14 at band q32, whereas the genetic locus of kappa light chain genes is on chromosome 2p11 and of lambda on chromosome 22q11 (Fig. 2). The variable domains of immunoglobulins are encoded by discontinuous gene segments that are separated from each other in the germline state (Fig. 2). The heavy-chain gene family consists of approximately 50 variable-region (V_H) genes that encode the first 95 amino acids of the variable portion of this peptide, more than 20 diversity-region (D) genes that encode a small number of amino acids, six joining-region (J_H) genes that encode the remaining 13 amino acids of the variable region, and nine functional constant-region (C_H) genes. The κ and λ gene families also contain a series of variable-region and joining genes located upstream from the constant-region gene or genes. As the pluripotent stem cell with its immunoglobulin genes in the separated germ-line configuration develops into an immunoglobulin-producing plasma cell, a process of DNA rearrangement occurs. This begins with activation of a heavy-chain gene by a rearrangement that combines a single D segment with a single J_H segment. Then a single V_K segment is combined with this DJ juncture. This rearrangement of a V gene segment with a D gene segment brings a promoter controlling sequence upstream from each V gene segment closer to a tissue-specific enhancer sequence that is between the J and C regions. This activates the gene complex, increasing transcription of mRNA for the heavy-chain gene, and leads to the production of cytoplasmic μ chain and, thus, the appearance of the pre-B cell. Surrogate light-chain genes, V pre-B and λ-5, are expressed without rearrangement during the earliest stages of B lineage differentiation. The products of these genes become associated with the μ heavy-chain gene product and appear on the surface of pre-B cells. Following effective heavy-chain gene rearrangement, there is a rearrangement of light-

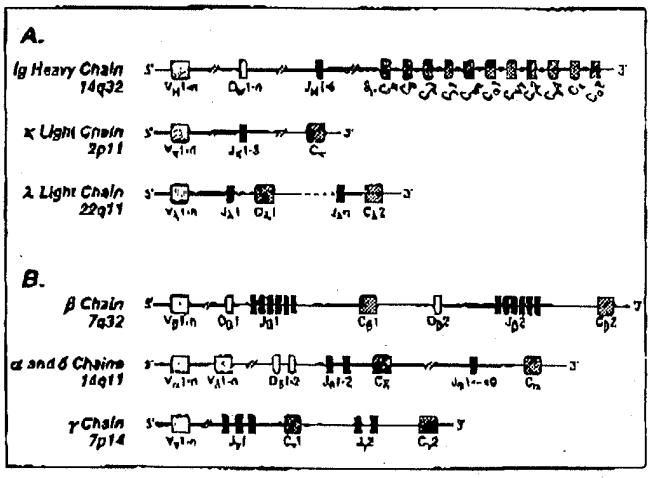

Fig. 2. (a) Genes of immunoglobulin chains and (b) T-cell receptor chains.

chain genes, beginning with rearrangements of the κ immunoglobulin locus with a recombination that juxtaposes one of many V_κ regions with one of the five J_κ segments to generate the complete transcriptionally active V_κ region. If efforts at generating a κ gene are not successful, activation of λ light-chain genes occurs. Following effective rearrangement of light-chain genes, the mature mRNA is translated, and IgM molecules can be produced and expressed on the cell surface, thus producing the immature B cell.

Although a B cell and its progeny produce only a single form of light chain, κ or λ, a B cell is capable of simultaneously producing IgM and IgD membrane forms of immunoglobulin and of switching, subsequently, to the production of other immunoglobulin isotypes. Establishing the order and structure of the heavy-chain constant region genes has helped to elucidate the mechanisms by which different classes are produced. The human immunoglobulin heavy-chain constant-region genes located on the long arm of chromosome 14 at band q32 are in the order shown in Fig. 2. The simultaneous production of IgM and IgD membrane forms, as well as the transition from membrane-bound receptors to a secreted form, involves alternative mRNA splicing. In contrast, the transition from a C_μ-expressing B cell to one expressing another isotype occurs by a phenomenon known as heavy-chain class switching or isotype switching. This is accomplished by the splicing of an area termed a switch region upstream from the μ heavy-chain gene, with the switch region 5' to the downstream heavy-chain gene to be expressed. Such recombination would result in a DNA rearrangement that is accompanied by deletion of the DNA between the switch region 5' from the C_μ gene and the switch region immediately 5' from the constant region to be used. This process of switching allows a new constant region to be transcribed with the pre-existing $V_H/D/J_H$ recombined gene. In addition, both membrane and secreted forms of the immunoglobulins may be produced by the same cell at different stages of differentiation. At a molecular level, the transition from the membrane to the secreted form involves alternative splicing of mRNA resulting in different mRNAs containing the secreted (Cμs) or membrane (Cμm) carboxy-terminal tail. Terminal differentiation of a B lymphocyte to a plasma cell forecloses these options so that a single plasma cell synthesizes and secretes an immunoglobulin of a single isotype and specificity (i.e. allelic exclusion).

The mature B-cell antigen complex is composed of an antigen-binding membrane immunoglobulin and the associated Igα/β proteins serving transducer/transporter functions. The transducer/transporter substructure is composed of disulfide-linked heterodimers of Igα (CD79a) and Igβ (CD79b) subunits. Igα is a product of the mb-1a gene and Igβ is encoded by the B29 gene. Thus the pre-B receptor complex involves a minimum of 10 chains: two Ig heavy chains, two Vpre-B chains, two λ5 chains and two Igα/β heterodimers. The CD19 complex that includes CD19, complement receptor 2 (CR2 or CD21), Leu-13 and TAPA-1 molecules may act as a coreceptor with the B-cell antigen receptor binding to the same antigen/complement complex.

The T-cell receptors for antigen are also heterodimers composed of either α and β or γ and δ subunits. The T-cell antigen receptor is associated with a cell surface complex of different non-polymorphic chains (CD3γ, CD3δ, CD3ε, CD3ζ). The arrangement of T-cell receptor genes is similar to that of Ig genes (Fig. 2). The T-cell receptor TCRβ chain locus is on chromosome 7q32-34, and the TCRγ chain on chromosome 7p14-15. The TCRα and δ genes are on chromosome 14q11. The TCRβ chain gene comprises discontinuous germ-line variable regions gene (Vβ) and duplicate sets of diversity (Dβ1, Dβ2), joining (Jβ1, Jβ2) and constant (Cβ1, Cβ2) gene segments. The TCRα gene consists of multiple variable (Vα) genes arranged in families, at least 40 joining (Jα) genes in a tandem array and a single 5' constant Cα gene. The TCRδ gene system composed of Vδ, Dδ, Jδ and Cδ segments is nested within the TCRα locus between the TCRα variable and TCRα joining region genes. The fourth gene family, the TCRγ family encoded by genes on the short arm of chromosome 7 (7p15), has many properties in common with other TCR genes, including assembly from diverse variable, joining and constant regions and rearrangement in T cells.

As with Ig genes, there appears to be a hierarchy in the rearrangement and expression of T-cell receptor genes. Rearrangement of the TCRγ and δ genes occurs early. If the rearrangements are effective, the γδ-T-cell receptor subunits together with the CD3 complex of proteins are expressed on the cell surface of Tγδ cells. In an alternative pathway of rearrangement, precursor cells initiate rearrangements of the TCRβ genes. In analogy with λ5-IgH pre-B-cell receptors there is a pre-T-cell receptor that is involved in the regulation of early T-cell development. The pre-T-cell receptor involves CD3 complex non-covalently associated with the TCRβ chain that in turn is disulphide linked to a pre-T-cell receptor α chain (pTα), a type I transmembrane protein that acts as a surrogate for the TCRα chain. Subsequently, TCRα genes are rearranged and expressed, permitting the production and cell-surface expression of the α/β heterodimer. The mature T-cell receptor heterodimers become associated with the CD3 complex which serves a transporter/transducer function. The receptor complex is then expressed on the surface of T cells. CD4 or CD8 act as coreceptors for the TCR by binding to the same MHC molecules as the TCR.

4 CYTOKINES AND CHEMOKINES

Immune responses as well as the effector phase of immune reactions are regulated by soluble mediators called interleukins or cytokines. Many cytokines and their receptors have been characterized in molecular form. Characteristic features of cytokines are their functional pleiotropy and redundancy, i.e. one cytokine shows multiple functions in a wide variety of tissues and cells and many different cytokines exert similar effects in the same cells. Cytokine producers are also multiple, i.e. many cytokines are produced by several different cells, and the production of cytokines is influenced by other cytokines, thus forming a 'cytokine network'. Major producers of cytokines in the immune system are monocytes and T cells.

Many cytokine receptors belong to the 'cytokine receptor family'. They have four conserved cysteine residues in their N-terminal region and 'Trp-Ser-X-Trp-Ser' motif external to the plasma membrane. These conserved residues are essential for maintaining the tertiary structure of the receptor molecules. Cytokine receptors do not have any unique sequences for signal transduction, such as tyrosine kinase, in their intracytoplasmic domain. Several cytokine receptors, such as IL-2Rα, IL-6R, IL-5R, GM-CSFR have very short intracytoplasmic domains, suggesting the presence of other chains for signal transduction. The IL-6 receptor system was shown to be composed of two polypeptide chains, an 80 kD IL-6R and a 130 kD signal transducer (gp130). Binding of IL-6 to IL-6R triggers an association with the 130 kD subunit which transduces the signal. gp130 has been shown to function as a signal transducer not only for IL-6 but also for LIF, Oncostatin M, IL-11 and ciliary neurotropic factor (CNTF) and cardiotropin (CT-1). Thus, this cytokine receptor system consists of two polypeptide chains, ligand specific receptor and common signal transducer. Recently, this concept is shown to

be applied to most other cytokine receptor systems. In the haematopoietic system, the receptors for IL-3, IL-5 and GM-CSF utilize a common β chain (βc) as a signal transducer. In the lymphoid system, a common γ chain (γc) is a shared element of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15. This is a reason why mutation in the gene encoding γc results in X-linked SCID in humans, whereas a disruption of the IL-2 gene in mice did not lead to a major effect on the development of T and B lymphocytes. IL-4 and IL-13 receptors share a common 140 kD heavy chain (IL-4Rα) which associates with the γc in IL-4R and with a novel IL-13 binding chain of the IL-13R.

A mechanism common to a very large number of cytokine receptors which consist of two or more chains involves the cross phosphorylation and activation of members of the Jak kinase family. Cytokine receptors associated with Jak family kinases include receptors for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15 as well as for GM-CSF, IFNα, IFNβ and IFNγ. Cytokine binding induces receptor chain heterodimerization. This results in cross phosphorylation on tyrosine residues and activation of the associate Jak kinases (e.g. Jak1 associated with IL-4R-α and Jak3 associated with γc). Activated Jak's phosphorylate tyrosine resides on one, or more, of the receptor chains. These P-Tyr residues then serve as docking sites for SH2 domain-mediated docking of STAT (signal transduces and activators of transcription) molecules, e.g. STAT6 in the case of IL-4R. Receptor-bound STAT molecules are in turn tyrosine phosphorylated. This leads to their dimerization through P-Tyr-SH2 chain interactions, uncovering nuclear localization signals. STATs then translocate to the nucleus where they bind to the DNA consensus sequence [TTC NNN (N) GAA] and participate in the transcriptional activation of cytokine responsive genes, e.g. Cε in the case of IL-4.

At present, 18 interleukins (IL-1 to IL-18) have been cloned and their biological activities in immune regulation are under intense scrutiny. Assays are available for estimation of cytokine levels.

4.1 IL-1

IL-1 is one of the typical examples of multifunctional cytokines. It is produced mainly by monocytes. IL-1α and IL-1β, which show only 24% (human) homology in their amino acid sequences, utilize the same IL-1 receptor, which belongs to the Ig-superfamily. A naturally occurring IL-1 inhibitor (IL-1ra) also shows a certain sequence homology with IL-1α and β and binds to the IL-1 receptor, but it cannot activate the signal pathway. Therefore, IL-1ra functions as a competitive inhibitor of IL-1. IL-1 is important for the early activation of T cells as a co-stimulatory factor. IL-1 is a strong inducer of IL-6 and several activities of IL-1 in immune regulation can be exerted through IL-6. IL-1 is one of the typical inflammatory cytokines and is involved in the generation of prostaglandins. Fever is generated by IL-1 through the generation of IL-6.

4.2 IL-2

IL-2 is a major T-cell growth factor. Activated T cells produce IL-2 and express high affinity IL-2 receptors and T cells proliferate in an autocrine or paracrine fashion. IL-2 is not needed for T-cell maturation as both humans and mice with IL-2 deficiency possess normal number of circulating T cells, which however fail to proliferate to mitogens and antigens unless IL-2 is added. IL-2 plays an important role in mature T-cell apoptosis (activation-induced cell death - AICD). The high affinity IL-2 receptor is formed by three polypeptide receptor components, IL-2Rα (Tac, CD25), IL-2Rβ, and γc and signals can be transduced through IL-2Rβ and γ chains. Activated B cells can express IL-2R and IL-2 induces growth and antibody production in such activated B cells. Resting NK cells express IL-2Rβ and γc. The IL-2Rβ chain associates with Jak1 and γc associates with Jak3. Engagement of the IL-2R results in phosphorylation and activation of STAT 5. Mutation in the γc chain gene and of Jak3 result in phenotypically similar severe combined immunodeficiency with absent T and NK cells, but circulating B cells.

4.3 IL-3

IL-3 is a multi-colony stimulatory factor (multi-CSF) and is involved in proliferation of early progenitors of haematopoietic cells. IL-3 exerts a synergistic effect with IL-6 on the expansion of early haematopoietic progenitors. IL-3 is produced by activated T cells. The IL-3 receptor comprises two polypeptide chains like the IL-6 receptor. The βc chain that functions as a signal transducer for the IL-3 receptor is common to IL-5R and GM-CSFR. These three receptors use the Jak1/Jak2 and STAT 5 pathway.

4.4 IL-4

IL-4 was originally identified as B cell stimulatory factor (BSF-1) and was shown to induce the early activation of resting B cells upon antigen exposure. IL-4 induces isotype switching of B cells into IgE producing cells. Anti-IL-4 inhibits IgE production in parasite-infected mice, indicating an essential role for IL-4 in IgE production. IL-4 knockout mice do not produce any IgE. IL-4 is shown to be a potent growth factor for mast cells and to induce FcεRII (CD23) on B cells and monocytes. These results strongly suggest the involvement of IL-4 in immediate-type hypersensitivity. IL-4 is produced not only by activated T cells but also by mast cells and basophils. IL-4 functions as a growth factor for T cells and is involved in autocrine and paracrine growth of activated T cells. The IL-4 receptor consists of a heavy 140kD chain which associates with Jak1 and of the γc chain which associates with Jak3 and acts as a signal transducer. IL-4R engagement leads to the phosphorylation and activation of STAT 6.

4.5 IL-5

IL-5 may enhance B-cell differentiation and also acts as an eosinophil differentiation factor. The IL-5 is mainly produced by activated T cells. The IL-5 receptor consists of two polypeptide chains like the IL-6R, one of which (βc) is a signal transducer of IL-5R that is common to GM-CSFR and IL-3R. IL-5 knockout mice fail to develop eosinophilia.

4.6 IL-6

IL-6 is the prototypic multifunctional cytokine. It was originally identified as a B-cell differentiation factor and is involved in the final maturation of B cells into antibody producing cells. IL-6 is essential to antibody production. It also activates T cells and haematopoietic progenitors. IL-6 induces maturation of megakaryocytes and induces thrombocytosis in inflammation. IL-6 is a major inducer of the acute phase reaction in inflammation. Excessive production of IL-6 has been shown in several autoimmune diseases. IL-6 is a potent growth factor for myeloma and plasmacytoma cells and appears to be involved in multiple myeloma and plasmacytomas. The IL-6R consists of an IL-6 binding chain and a signal transducing gp130 chain shared by receptors LIF, CNTF and OSM. The IL-6 uses the Jak1/Jak2 and STAT3 pathway. IL-6 is produced by a wide variety of cells, but mainly by monocytes. IL-1 and TNFα are strong inducers of IL-6 in monocytes. Anti-viral antibody response was

5–10-fold reduced in IL-6 knockout mice. Furthermore, IL-6$^{-/-}$ mice were defective in their mucosal IgA response. The inflammatory acute phase reaction is severely compromised in IL-6$^{-/-}$ mice; optimal responses to trauma and infection can only be mediated in the presence of IL-6.

4.7 IL-7

IL-7 is a major B-cell lymphopoietin and is involved in the growth and differentiation of pre-B cells. It also acts as a growth factor for thymocytes and mature CD4+ and CD8+ cells. IL-7 is produced by stromal cells in the marrow, thymus and spleen. IL-7R utilizes γc in signal transduction. Lymphoid development is severely impaired in IL-7$^{-/-}$ and IL-7R$^{-/-}$ mice.

4.8 IL-8

IL-8 is an inflammatory cytokine produced by monocytes and involved in neutrophil chemotaxis. Several other cytokines, such as Platelet Basic Protein (PBP), Platelet Factor 4 (PF4), γ-Interferon Inducible Protein (IP10) and Growth Related Gene (Gro), show sequence homology with IL-8. IL-8, PBP, PF4, IP10 and Gro belong to the CXC family of chemokines. The other family of chemokines is the CC family. In contrast to CC chemokines which have two adjacent cysteines; CXC chemokines have the same two cysteines separated by an amino acid. The list of chemokines is currently expanding with more than 20 family members some of which overlap in function, receptor utilization and target specificity. Recently the CCRK5 receptor was shown to be shared by the CC chemokines Rantes, Mip1-α and Mip1-β and to function in monocytes as a co-receptor with CD4 for the HIV gp120. In parallel the CXC chemokine receptor fusin (CXCR3) is the co-receptor with CD4 for HIV entry into T cells. The CXC chemokine that uses CXCR3 as its receptor is SDF-1/PBSF. This stromal derived factor/pre-B cell stimulatory factor shows synergy with IL-7 for B-cell development. Knockout of SDF-1/PBSF results in failure of B-cell development.

4.9 IL-9

IL-9 is identified as a T-cell growth factor distinct from IL-2 or IL-4. It is produced by CD4+ helper T cells and acts on helper T cells but not on CD8+ cytotoxic T cells. IL-9 was shown to act on mast cells stimulating their growth in a manner similar to IL-4. IL-9R utilizes γc in signal transduction.

4.10 IL-10

IL-10 was originally called CSIF (Cytokine Synthesis Inhibitory Factor), which is produced by monocytes and Th2 cells. It inhibits the production of cytokines by Th1 cells. As with other cytokines, IL-10 also exerts pleiotropic functions and induces growth of T cells and mast cells. IL-10 is produced not only by Th2 cells but also by B lymphoma cells, macrophages and mast cells. The IL-10 receptor is composed of two chains and uses the Jak1/Tyr2 and STAT 3 pathway.

4.11 IL-11

IL-11 is identified as a plasmacytoma growth factor and has the pleiotropic functions of IL-6 and its receptor shares the gp130 of the IL-6R.

4.12 IL-12

IL-12 is a heterodimer of glycoproteins, p35 and p40, which acts on B cells, NK cells and monocytes to induce proliferation and cytokine synthesis, especially of interferon-γ. IL-12$^{-/-}$ mice show increased susceptibility to leishmaniasis. IL-12R uses the Jak2/Tyr2 and the STAT4 pathway.

4.13 IL-13

IL-13 is produced by Th2 and mimics the effects of IL-4 on IgE production. The IL-13R is expressed on human B cells, T cells and monocytes. It uses a unique IL-13 binding chain which associates with Jak2 and shares with the IL-4R, the 140kD IL-4Rα chain which associates with Jak1. IL-13 activates STAT-6.

4.14 IL-14

IL-14, formerly called high molecular weight B-cell growth factor, has been reported to enhance growth and differentiation of B cells. The existence of this cytokine has been challenged.

4.15 IL-15

IL-15 acts on activated T cells, B cells, and on NK cells to induce proliferation and differentiation. Its major receptor includes IL-2Rβ and γc as well as a unique IL-15R α chain. An alternative IL-15 receptor is found on mast cells.

4.16 IL-16

IL-16 is a CD4+T lymphocyte attractant and competence growth factor which uses CD4 as a receptor. CD8+ T cells serve as a source of IL-16.

4.17 IL-17

IL-17 is a glycoprotein of 155 amino acids secreted as an homodimer by activated memory CD4+ T cells, and is highly homologous to Herpesvirus Saimiri gene 13. IL-17 stimulates epithelial, endothelial and fibroblastic cells to secrete cytokines such as IL-6, IL-8 and granulocyte-colony-stimulating factor, as well as prostaglandin E_2. Furthermore, when cultured in the presence of IL-17, fibroblasts could sustain the proliferation of CD34+ haematopoietic progenitors and their preferential maturation into neutrophils.

4.18 IL-18

IL-18 is a novel cytokine produced by liver cells that possesses potent biological activities, including the induction of IFN-γ production by spleen cells and the enhancement of NK cell cytotoxicity. In addition, IL-18 augments granulocyte-macrophage-CSF production and decreases IL-10 production by Con A-stimulated PBMC.

4.19 Other cytokines

In addition to the interleukins and their receptors, other cytokines and monokines affect the immune system. Interferon-γ, secreted by activated T cells, is the most important cytokine in the induction of MHC class II molecule expression. TNF-α is a prominent monokine that shares many functions of IL-1, except for the induction of IL-2. TNFαR are of two types. TNFRI mediates cell death and uses signal transducer molecules that contain death domains. TNFRII delivers growth signals and activates NFκB. It uses TRAF1 and TRAF2 molecules as signal transducers. Lymphotoxin α–β is important for the normal development of lymphoid architecture (in mice). The colony stimulating factors, such as GM-CSF, G-CSF and M-CSF, act in addition to IL-3, as growth factors for immunologically relevant cells. The interferon α, β and γ receptors use the Jak/STAT signalling pathway. Deficient interferon-γ receptor due to mutations in the α chain in the receptor results in undue susceptibility to intracellular pathogens that resides in macrophages and in fatal

mycobacterial infection. Immunity to infection with *Listeria monocytogenes* is severely compromised in TNFR$^{-/-}$ as well as interferon-γ$^{-/-}$ and IL-6$^{-/-}$ mice, indicating an important role of these cytokines in innate immunity. Transforming growth factor β (TGFβ, a cytokine produced by many cells) has been identified as a major suppressor of immune function and is involved in class switching to IgA.

5 ANTIGEN PRESENTATION, CELL ADHESION AND SIGNAL TRANSDUCTION

Antigens are taken into antigen presenting cells (APC) by receptor mediated endocytosis (via C3 or Ig receptors) or by fluid phase endocytosis. Protein antigens, once in the phagolysosome, are digested by proteolytic enzymes. Antigenic fragments are then shunted to a specialized compartment for peptide loading. In this compartment, antigenic peptides bind to class II histocompatibility molecules and the complex thus formed is transported to the cell surface of the APC. For the most part, cells of monocyte-macrophage lineage and mature B cells serve as APC.

For antigens within the cell including many viral antigens, there is degradation of the proteins in part by proteasomes followed by transport of the resultant peptides into the endoplasmic reticulum where they bind to class I MHC. This transport across the Golgi membrane is mediated by members of the ATP binding cassette (ABC) superfamily of transporters (e.g. TAP-1 or TAP-2), which are encoded by genes within the MHC. Mutations in TAP-2 are associated with MHC class I deficiency.

The interaction of APC with T cells as well as T–B-cell collaboration are facilitated by adhesion molecules. The ligands and counter-ligands of many of these adhesion molecules have now been well defined (see Fig. 3). ICAM-1 and LFA-1 are reciprocally interacting; both molecules are found on T cells as well as on APC. LFA-1 is defective in leukocyte adhesion deficiency (see 12.2.1) and the CD40 ligand is defective in the hyper IgM Syndrome (see 9.2.2).

The interaction of the T-cell receptor (TCR)-CD3 complex with presented antigen results in signal transduction that leads to phosphorylation of CD3 and the activation of T cells (see Fig. 4).

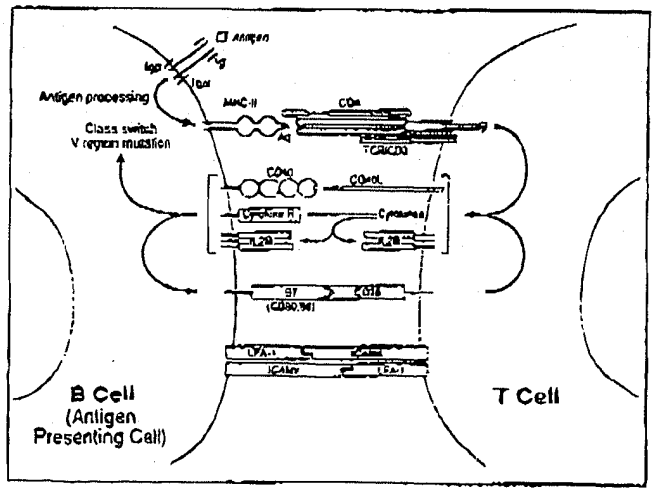

Fig. 3. Adhesion molecules and their ligands.

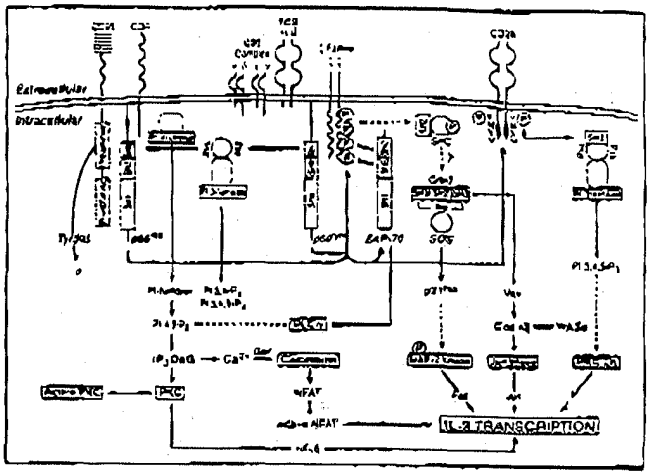

Fig. 4. Signal transduction pathway in T cells. CD3 is indiated in the T-cell lineage by 5 bars and Igα and Igβ of the B cell receptor by two bars.

Upon TCR cross linking, the src type protein tyrosine kinases (PTK), p56^{lck} and p59^{fyn}, respectively, associated via their N terminal domains with CD4 and CD3ζ, phosphorylate CD3ζ in tyrosine residues of the Immune Tyrosine Activation Motif (ITAM), Y-X-X-L-X(7-8)-Y-X-X-L/I. Three ITAMs are present in CD3ζ. Activation of lck and fyn also critically depends on the transmembrane phosphatase CD45, which dephosphorylates the carboxy-terminal autoinhibitory tyrosine residue of these src kinases. The phosphorylation of CD3ζ initiates an activation cascade by enabling the TCR to recruit downstream molecules. Thus, phosphorylated CD3ζ is now able to bind the PTK, ZAP-70, and the adaptor protein, Shc, via interaction between phosphotyrosine residues on CD3ζ and SH2 domains in these proteins. Receptor-bound ZAP-70 can phosphorylate substrates such as phospholipase C-γ (PLC-γ) and recruit them to the receptor complex. The other CD3 components, each of which contains one ITAM motif, may also serve as substrates for p56^{lck} and p59^{fyn} and, as demonstrated for CD3ε, may bind ZAP-70 and thus may function as additional independent signalling units.

Phosphorylated PLC-γ recruited to the membrane breaks down membrane inositol phosphatides, mainly PI4,5-P2, to generate the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3), which respectively activate protein kinase C (PKC) and mobilize Ca^{++} from intracellular stores. Released Ca^{++} plays a critical role in the activation of downstream enzymes, which include the Ca^{++}/calmodulin dependent phosphatase, calcineurin. Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) allowing its translocation to the nucleus. Several isoforms of NFAT exist of which NFATc is the most critical for T-cell activation. Grb-2 interacts with, among other things, Vav, which is phosphorylated by ZAP-70 following TCR receptor engagement. Vav is a GTP exchange factor that activates the GTPase, Cdc42, a member of the rho family of proteins. In its GTP bound state, Cdc42 binds Wiskott–Aldrich syndrome protein (WASp) and activates the Jun kinase pathway. Jun and Fos form a heterodimer that can bind to specific DNA sequences as well as to NFAT to enhance its transcriptional activity.

Another activation pathway recruited by aggregation of the TCR is the p21^{ras} pathway. Phosphorylated CD3ζ has been reported to bind to the SH2 domain-containing adaptor protein, Shc. Shc protein activates p21^{ras} by means of the intermediate adaptor protein Grb-2.

consisting of two SH3 and one SH2 domains, and the guanine nucleotide releasing protein SOS, which is capable of exchanging GDP with GTP leading to the conversion of $p21^{ras}$ to an activated GTP bound state. This in turn activates the MAP kinase pathway, which culminates in nuclear fos/jun expression.

In addition to the tyrosine phosphorylation cascade, the activated lipid kinases, PI3 kinase and PI4 kinase, are recruited to the activated TCR. PI3 kinase is recruited by association of its p85 non-catalytic subunit with the SH3 domain of $p56^{lck}$, $p59^{fyn}$ or ZAP-70. PI3 kinase phosphorylates the D-3 position of the inositol ring of phosphatidylinositol leading to the generation of PI3,4-P2 and PI3,4,5-P3. PI,3,4,5-P3 activates PKC isozymes, e.g. PKCζ which play an important role in cell survival and division.

Activation of PKC, calcineurin and ras and Cdc42 result in the activation and expression of a number of transcription factors that include respectively NFκB, NFAT, Fos and Jun. These factors are critical for the transcription of *IL-2* and *CD40L*. The expression of these genes leads to T-cell activation and proliferation.

Optimal *IL-2* gene expression requires, in addition to TCR cross-linking, engagement of the costimulatory molecule CD28 by its counter-receptor B7 (CD80 and CD86) on APCs. CD28 contains a YMXM motif, which is a potential target for phosphorylation by PTK that has been activated following TCR cross-linking. The phosphorylated motif recruits PI-3 kinase via the SH2 domain of its non-catalytic subunit. PI-3 kinase activated via CD28 synergizes with enzymes activated via the TCR to enhance IL-2 production. Activated T cells express another B7 ligand CTLA-4, which in contrast to CD28, delivers a signal that terminates T-cell activation.

The B-cell antigen receptor signals in ways similar to the TCR. Surface Ig is associated with Igα and Igβ subunits each of which has an ARAM motif. The src kinases lyn, blk and the ZAP-70 homologue syk associate with the sIg receptor. The B-cell specific molecule CD19, which associates with the Ig receptor, contains, in its cytoplasmic tail, the YXXM motif. This mediates recruitment of PI-3 kinase.

The role of various enzymes and pathways is illustrated by several ID diseases, and by knockout mice. Disruption of $p56^{lck}$ leads to abnormal thymic maturation. Disruption of $p59^{fyn}$ affects only the function of peripheral T cells. ZAP-70 deficiency in humans results in CD8 deficiency and in deficient CD4+ T-cell function. In mice, ZAP-70 deficiency results in deficiency of both CD4 and CD8 cells. CD3γ and ε chain deficiency results in severely impaired T-cell receptor expression and variable impairment in T-cell function. NFAT abnormality leads to deficiency in the production of IL-2 and other cytokines.

6 ASSESSMENT OF PATIENTS SUSPECTED TO HAVE A PRIMARY IMMUNODEFICIENCY

6.1 Patient selection and identification

Early diagnosis and treatment of patients is vital. Many Immunodeficiency Diseases (ID), both primary and secondary, are treatable. Early treatment may prevent the otherwise inevitable devastating damage that can occur. Thus, whenever persistent or recurrent infections occur, which do not respond as expected to antibiotics, or which are caused by unusual or opportunistic infectious agents, primary or secondary ID must be considered. This is particularly important if family members have died in infancy or have similar susceptibility to infections. When such patients are encountered, studies should be carried out that permit identification of ID. Families of patients with ID should also be investigated.

Such patients can be divided into seven main groups: (a) infants from families known to have hereditary ID: prenatal diagnosis (see Section 8) is possible in many instances; (b) infants whose siblings have a possible or an established ID; (c) infants with syndromes or other diseases known to be associated with ID (see Section 10); (d) infants who fail to thrive, have unusually persistent infections with low virulence or opportunistic agents, unusual rashes, or persistent diarrhoea (see Section 9.2, Combined ID); (e) patients with recurrent or persistent infections that fail to respond as expected to antibiotic therapy; recurrent sinopulmonary infections are commonly the presenting problem (see Section 9.3, Predominantly antibody defects), patients with chronic obstructive pulmonary disease should be investigated for ID; (f) patients with recurrent skin infections, abscesses, periodontitis, or unusual wound healing (see Section 12 and Table 5; Defects of phagocytic function); (g) patients with recurrent neisserial infections or with systemic lupus erythematosus (see Section 11 and Table 4; Complement deficiencies).

Normal neonates and young infants have higher lymphocyte counts than older children and adults. Lymphopenia in young infants should prompt investigation of ID. Normal or increased numbers of lymphocytes in babies and young children do not exclude the possibility of primary immune deficiency, nor even the possibility of SCID or CID. These cells may be of maternal origin, even if graft vs. host (GvH) disease is not obvious. The presence of normal or increased IgM concentrations with absence of other isotypes in young children is not limited to hyper-IgM syndrome and may be found in X-linked SCID and other forms of combined immune deficiency diseases. If a child in whom the possibility of a primary defect of cell mediated immunity is suspected had been vaccinated post partum with BCG, tuberculostatic treatment should be started immediately.

The necessary screening for ID requires assessment of the patient's ability to develop and express B-cell, T-cell or combined T- and B-cell immunological functions. The biological amplification processes (complement, cytokines, etc.) and the basic effector mechanisms (phagocytosis and the inflammatory response) need to be investigated.

Evaluation should begin with enumeration of the crucial cell populations, T cells, B cells, granulocytes, monocytes; quantitative measurement of serum immunoglobulin concentrations of IgM, IgG, and in some instances IgA and IgE.

Immunological competence can be further assessed by quantitation of specific antibody responses to ubiquitous antigens as well as to immunization with well tolerated, commercially available antigens (e.g. tetanus and diphtheria toxoids, killed polio antigens, and haemophilus conjugates). Polysaccharide vaccines are obtainable; quantitative responses are difficult to evaluate because age, sex, ethnicity and race adjusted standards are not available. T-cell-mediated immunity can be determined by skin testing for delayed hypersensitivity with a battery of antigens which in the aggregate yield positive responses in a high proportion of healthy individuals, but may be difficult to assess in infants and younger children because such immunity has not yet been acquired. T-cell immunity can also be evaluated by *in vitro* responses of peripheral blood lymphocytes to phytomitogens, common antigens, and/or anti-CD3.

In those patients with relevant symptoms (see Section 11) total haemolytic complement and complement components of both the classical and alternative activation pathways should be measured immunochemically and functionally.

Patients suspected of having chronic granulomatous disease should have phagocytic function evaluated by: (a) semi-quantitative nitro blue tetrazolium dye reduction after exposure of patient's peripheral blood cells to a phagocytic stimuli; (b) measurement directly of O_2^- radical formation after stimulation of patient's blood cells using PMA or dichlorfluorine; and quantitatively measuring O_2^- production; (c) by measuring the phagocytic response using chemiluminescence following similar stimulation; (d) by quantifying capacity of patient's cells to ingest and kill catalase positive micro-organisms such as staphylococci or paracolobacilli. The integrity of the inflammatory response can be tested by Rebuck skin window techniques. *In vitro* analysis of the inflammatory response can be studied by measurement of chemotaxis, chemokinesis, and the capacity to produce and release selected inflammatory cytokines. Expression of adhesion molecules (e.g. CD18) should be assessed.

Diagnosis of several IDs can be performed at the level of the respective genes responsible for the immunodeficiency. Analysis of the gene at the molecular level as well as demonstration of the respective gene products may be performed in specialized laboratories.

6.2 *Immunoglobulins and antibodies*

6.2.1 Measurement of immunoglobulin concentration. Serum immunoglobulins are commonly measured by radial immunodiffusion or automated immunoturbidometric methods. Other techniques such as radioimmunoassay, and ELISA are also available and useful for IgE and IgD measurements. Quality assessment (QA) is widely available throughout the world to ensure reliability. Electrophoresis and immunoelectrophoresis are not satisfactory techniques for the quantitation of immunoglobulins. Immunoelectrophoresis and immunofixation are however useful in the detection of M-components. Immunoglobulin can also be measured in body secretions, e.g. saliva, tears and milk but this is rarely indicated. Monomers of IgM (usually a pentamer) are present in serum of some ID patients such as CVID and hyper IgM and may give spuriously high IgM levels. The subclasses of IgG can be measured by simple radial diffusion or ELISA methods. Although standards and normal values for IgG subclasses are now in use, the ranges in normal children are very wide and do not take into consideration genetic and geographical variations. IgG subclass determination is of limited value in assessing patients with clinical immunodeficiency, since functional antibody deficiency may be present despite normal IgG subclass levels, and conversely deficient levels of a single subclass of IgG may be found in individuals who have effective specific antibody production and are clinically normal. Methods for IgA subclass determinations are not yet readily available and their measurement is not yet of value. Serum immunoglobulin concentrations vary with age and environment: thus appropriate regional and ethnic age-related and sex-related norms must be used.

Concentrations of immunoglobulins cannot be used as the sole criteria for the diagnosis of primary ID. Diminished immunoglobulin levels may be due to loss as well as decreased synthesis. An indirect indication of loss may be obtained by measuring serum albumin, which is usually lost concomitantly (e.g. through the gastrointestinal or renal tracts). Limited heterogeneity of immunoglobulins and abnormal kappa-lambda light-chain ratios have been observed in ID syndromes.

6.2.2 Assessment of antibody formation following immunization. Antibody-mediated immunity (humoral immunity) may be assessed by antibody responses to those antigens to which the population is commonly exposed, or following active immunization. Protein or polysaccharide antigens may be used; the latter are particularly relevant in patients with sinopulmonary infections. It is important that sensitive and reproducible assays are used, such as ELISA. Normal ranges of IgG antibodies in children following immunization are available but need careful interpretation.

Live vaccines (e.g. BCG and vaccines for poliomyelitis, measles, rubella and mumps) should never be given, even to family members, when primary ID is suspected. BCG is contraindicated in patients with T-cell or phagocytic cell IDs. Live viral vaccines, including polio, measles, mumps and rubella vaccination must not be given in patients with T-cell ID (as defined by an absence of antigen specific response, e.g. TT) and XLA or other severe B-cell deficiencies. Live vectors are also contraindicated in patients with ID.

The following tests are recommended:

1 'Natural' antibodies: A and B isohaemagglutinins are sometimes used as measures of IgM antibodies.

2 IgG antibody responses to common immunizations: (a) in unimmunized children, commercial diphtheria/tetanus (DT) or Hib-conjugate vaccines may be given in recommended doses. Blood is taken 3 weeks after the last immunization and IgG antibodies determined usually by ELISA. A Schick test may be performed for diptheria antibodies. Three doses of killed poliomyelitis vaccine (1.0 ml intramuscularly, at intervals of 2 weeks) can also be used; blood is taken 2 weeks after the last injection and antibody determined, usually by virus neutralization; (b) in patients who have been immunized with diphtheria/tetanus (DT) or diphtheria/pertussis/tetanus (DPT) vaccine, IgG antibodies are measured; if low, one booster injection is given, followed by measurement of antibodies and/or a Schick test. The widespread routine use of *Haemophilus influenzae* type b (Hib) conjugate vaccine makes measurement of IgG antibodies to Hib valuable if this immunization has been completed.

3 Additional active immunizations that may be recommended: (a) bacteriophage ϕX 174, a bacterial virus that is not infective for humans, has been shown to be a potent, safe and useful antigen; it allows measurement of antigen clearance and primary and secondary immune responses[a]; (b) to measure IgG antibody responses purely to carbohydrate antigens[b], pneumococcal or meningococcal polysaccharides, or Haemophilus b polysaccharide free of carrier proteins, can be used as well as typhoid-Vi antigen. Blood is drawn after 3 weeks and IgG antibody is determined by ELISA. These and other pure polysaccharide antigens are not useful in infants under 2 years of age. Interpretation of results in children under 5 years old is difficult, as the age at which these responses develop ranges from 2 to 4 years of age; (c) other useful antigens to measure primary response include: (i) tick-borne encephalitis (killed) vaccine[c]; (ii) hepatitis A vaccine. Hepatitis B is not a reliable antigen for testing immune competence because of the high frequency of non-responders in the population, particularly in patients over 40 years of age.

6.2.3 B lymphocytes. B lymphocytes are counted by detection of the membrane-bound CD antigens CD19 and CD20. This can be done either by using a flow cytometer and fluorescent-labelled monoclonal antibodies to B-cell antigens or by immunohistological

techniques on whole blood films. Monocytes can be distinguished from B lymphocytes by gating on a flow cytometer, by peroxidase or esterase staining on films, ingestion of IgG-coated latex particles or by monoclonal antibodies specific for monocytes, such as to CD14.

Pre-B cells may be identified among bone marrow cells with purified fluorochrome labelled antibodies to detect cytoplasmic μ heavy chains in CD19+ cells.

6.3 *Cell-mediated immunity (CMI)*

A number of tests are commonly employed for assessing CMI, including: delayed-type skin reactions; enumeration of T cells and T-cell subsets; *in vitro* tests of T-cell function.

6.3.1 Skin testing. Delayed cutaneous hypersensitivity (DCH) is a localized immunological skin response; the prototype is the tuberculin skin test. Because DCH is dependent on functional thymus-derived lymphocytes (T lymphocytes), DCH may be used in screening for T-cell mediated immunodeficiency. Antigens generally used are: mumps, trichophyton, purified protein derivative (PPD), candida or monilia, tetanus or diphtheria toxoids. To ascertain defective CMI several antigens must be used. All skin tests are done by intradermal injection of 0.1 ml of antigen. Results should be read in 48–72 hours for the maximal diameter of induration, which indicates intact cell mediated immunity. Erythema is not an indication of DCH.

1 Tuberculin: 0.1 ml containing 2 to 10 international units (IU) of Tween stabilized soluble PPD.
2 Candida or monilia[a]: Initially test at 1:100 dilution. If no reaction, test at 1:10 dilution.
3 Trichophyton[a]: use at 1:30 dilution.
4 Mumps[a]: use undiluted; read at 6–8 hours for early Arthus reaction (antibody mediated) and then at 48 hours for DCH.
5 Tetanus and diphtheria fluid toxoids[b]: use at 1:100 dilution.

A positive DCH is informative while a negative DCH test may be difficult to interpret. This is because DCH is influenced by age, steroid therapy, severe illness and recent immunization.

We do not recommend the use of dinitrochlorobenzene (DNCB) for skin testing; it is mutagenic and can cause necrosis. We also do not recommend the use of any multitest system for assessing CMI.

6.3.2 T lymphocytes. Because of the reliance on the phenotypic designation of T-cell subsets in evaluating patients with ID, it is essential to understand the normal differentiation and functions of these cells (see Section 2).

T cells can be enumerated by immunofluorescence with the use of monoclonal antibodies to CD3. Monoclonals to CD3 enumerate NK as well as T cells. Flow cytometry techniques are more reliable, reproducible and sensitive than visual microscopic enumeration; if a flow cytometer is not available, immunohistological techniques using either enzymatic or immunofluorescent labelled antibodies can be used. CD4 and CD8 monoclonal antibodies recognize important subsets of T cells, though monoclonal antibodies to CD8 enumerate NK as well as T cells. CD4 cells recognize antigen in association with the class II MHC (HLA-D) molecules, and CD8 cells recognize antigens in association with class I MHC (HLA-A, HLA-B and HLA-C) molecules. Antigen-specific T-cell responses are MHC restricted. Abnormalities in the number of CD4 or CD8 cells can be associated with abnormalities in cognate as well as regulatory functions of T cells and may lead to immunoincompetence or auto-immunity.

In suspected cases of hyper IgM immunodeficiency, T cells activated by PMA and ionomycin should be analyzed for expression of the CD40 ligand.

6.3.3 In vitro stimulation of lymphocytes. Lymphocytes can be activated *in vitro* by (a) mitogens such as phytohaemagglutinin (PHA) pokeweed mitogen (PWM) or concanavalin A (Con A); (b) antigens such as PPD, candida, streptokinase, tetanus and diphtheria, if the patient has had prior encounter with the antigen or with superantigens such as toxic shock syndrome toxin (TSST); (c) allogeneic cells; and (d) antibodies to T-cell surface molecules involved in signal transduction such as to CD3, CD2, CD28 and CD43.

T-lymphocyte activation can be assessed directly by (a) expression of activation antigens; (b) measuring blastogenesis and/or proliferation of cells; and (c) release of mediators.

A rapid result can be detected by detection of activation markers. Activated T cells express CD69, IL-2 receptors alpha (CD25), transferrin receptors (CD71) and MHC class II molecules not present or present in low numbers on resting T cells. T-cell populations to be assessed for their capacity to express these receptors are stimulated with a soluble lectin such as PHA, and examined 1–2 days later by direct or indirect immunofluorescence using monoclonal antibodies to CD25, or transferrin CD71 or MHC class II molecules and a flow cytometer.

The blastogenic response is assayed after 3–7 days depending on the nature of the stimulant, by ^{3}H- or ^{14}C-labelled thymidine incorporation for 16–24 hours. This is followed by DNA extraction or cell precipitation on filter paper and subsequent liquid scintillation counting. Control values of unstimulated cultures vary from person to person and from day to day. Data on unstimulated and stimulated cultures should always be given. Soluble PHA or Con A require the presence of monocytes for stimulation of T cells; under certain conditions, however, such as when bound to particulate matter, they may also stimulate B cells. PWM stimulates a response to both T and B cells, although T cells must be present for the B cells to be stimulated. The mixed lymphocyte reaction (MLC) results from T-cell reactivity to MHC antigens displayed on B cells and monocytes. It should be noted that, when normal irradiated or mitomycin C-treated lymphocytes are the stimulators of an MLC, the normal T cells in the culture may secrete factors that induce blastogenesis in the patient's lymphocytes. Therefore it is preferable to use B-cell lines or T-cell depleted normal cells as the stimulators.

Activated T-cells and monocytes synthesize and secrete interleukins-2,4,5 and 6, interferon-γ and other cytokines. The supernatants of peripheral blood mononuclear cells stimulated by soluble PHA can be assessed for IL-2 by an ELISA technique or by determining their capacity to stimulate ^{3}H-thymidine uptake by mouse IL-2 dependent cultured T-cell lines (e.g. CTLL2). The bioassay should be confirmed with blocking antibodies to IL-2, since other cytokines (e.g. IL-15) also activate this system. Specific *in vitro* systems also exist to assay other cytokines. It may soon be possible to measure synthesis of intracellular cytokines reliably and quantitatively.

6.4 *NK cells*

Monoclonal antibodies against CD16, CD56 and CD57 even though they are not lineage specific, may be useful for the detection and enumeration of natural killer (NK) cells. NK functional activity can be assessed by a cytotoxicity assay against cell line such as K562. This is important in the diagnosis of SCID, Chediak–Higashi syndrome and rare cases of isolated NK cell deficiencies.

7 OTHER TESTS

Examination of blood is essential, and biopsies of bone marrow, rectum and intestine, skin and lymphoid tissue may also be warranted for the diagnosis or classification of ID. In addition, postmortem examination may permit diagnosis of familial defects, important for genetic counselling and for understanding the pathogenesis of ID.

7.1 Blood

A total lymphocyte count is essential in the diagnosis of primary ID. Most patients with severe combined ID (SCID) and thymic hypoplasia have persistently low total lymphocyte counts (less than 1.5×10^9/l or 1500/mm^3). Lymphopenia can also be secondary to viral infections, malnutrition, cell loss, autoimmune diseases and myelophthisis as in haematopoietic malignancy. Normal lymphocyte counts do not exclude the diagnosis of SCID. Lymphocyte counts are variable in other forms of ID. Patients with reticular dysgenesis have pancytopenia. Thrombocytopenia and small platelets in a male infant suggest the Wiskott–Aldrich syndrome. Some patients with ID are anaemic; this may include a Coombs' positive haemolytic anaemia.

7.2 Bone marrow

Bone marrow aspiration or biopsy is important for exclusion of other diseases, for identification of plasma cells and pre-B cells and for diagnosis of obscure infections.

7.3 Lymph nodes

Lymph node biopsy is not necessary for the diagnosis of most ID but may be helpful. Rapidly enlarging lymph nodes should be biopsied; infection, malignancy or follicular hyperplasia may be the cause. Lymph node biopsies are potentially hazardous in SCID: they heal poorly and may produce a portal of entry for infection.

7.4 Rectal and intestinal biopsy

Examination of rectal tissue for plasma cells and lymphoid cells by histological and immunohistological methods may be informative in patients with common variable ID and selective IgA deficiency. Lymphoid cells are found in rectal and intestinal biopsies in normal infants aged more than 15–20 days old. Jejunal biopsy may show villous atrophy and may demonstrate *Giardia lamblia* and cryptosporidial infections.

7.5 Skin biopsy

Biopsy of skin is useful to establish a diagnosis of graft-versus-host (GVH) reaction in patients with ID after blood transfusion, bone marrow and fetal tissue transplantation or from maternal/fetal transfer of lymphocytes *in utero*.

7.6 Thymus

Thymic biopsy should be performed only by skilled surgeons. It should be performed only when thymoma is suspected.

7.7 Chimerism

Chimerism (the occurrence in one individual of two genetically different cell lines), when observed in ID can be congenital or acquired. The former is due to intrauterine implantation of maternal cells; the latter can occur after blood transfusion, bone marrow transplantation or fetal tissue implants. The presence and origin of lymphoid chimeric cells can be ascertained by karyotype, human leukocyte (HLA) or other antigenic typing, and analysis of highly polymorphic markers.

7.8 Special studies

Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) levels should be determined in all patients with possible SCID and T-cell deficiency. Serum alpha fetoprotein levels may be of value in separating patients with ataxia-telangiectasia (A-T) from those with other neurological disorders; it is increased (40–2000 μg/l) in at least 95% of persons with A-T. In patients with SCID, blood mononuclear cells should be examined for the presence of class II histocompatibility molecules to rule out the diagnosis of MHC class II deficiency. Cytogenic analysis is useful in diagnosis of A-T and other chromosomal breakage syndrome. Molecular cytogenetic studies are helpful in diagnosis of the DiGeorge anomaly and should be performed. This may be informative in other conditions (see Section 10).

7.9 Studies for infectious agents

The diagnosis of infection in ID is complex and beyond the scope of this report. In patients with ID, diagnosis of viral infection by antibody determinations are of little or no value since the patients have defective antibody formation. Direct viral isolation and/or identification of the viral genome (e.g. by PCR) are necessary to prove infection. In the presence of CNS symptoms, CSF cultures as well as PCR tests are important and brain biopsy may be required. HIV can be detected in peripheral blood lymphocytes and plasma by PCR analysis. Bronchial lavage may be useful for the diagnosis of *Pneumocystis carinii* and other pulmonary pathogens.

8 GENETICS CARRIER DETECTION AND PRENATAL DIAGNOSIS

Inheritance patterns are known for most of the primary immunodeficiency diseases and are given in Tables 1, 2, 3, 4 and 5. The known chromosomal map locations of several immunodeficiencies are given in Table 6. Table 4 contains the presently known chromosomal map locations of the complement genes. These recent advances in the precise mapping of the various immunodeficiency diseases and the availability of highly polymorphic markers often makes carrier detection and prenatal diagnosis possible (Table 7).

Carrier detection can be accomplished in several of these diseases. Where the location of the gene has been reasonably established (see Table 6), polymorphic markers may, in informative families, identify carriers with reasonable certainty. In those instances where a specific enzyme or complement component defect is present, heterozygote carriers can be ascertained from reduced levels of the enzyme or component in question. In some X-linked diseases, preferential selection against cells carrying the abnormal X chromosome during cell proliferation and differentiation of affected cell lineages permits determination of the carrier status. Preferential selection does not occur in carriers of the X-linked hyper IgM syndrome or X-linked chronic granulomatous disease.

At the present time, prenatal diagnosis can be made by obtaining fetal blood samples, amnion cells or chorionic villus biopsy. In some ID polymorphic markers can be used to establish diagnosis prenatally. The absence of B or T lymphocytes from umbilical cord blood can be used for the prenatal diagnosis of X-linked agammaglobulinaemia and SCID, respectively. However, whenever possible, prenatal diagnosis should be accomplished by molecular tests. Chronic villi are preferable to fetal blood samples to ascertain adenosine deaminase or purine nucleoside phosphorylase deficiency. Absence of cell membrane components such as MHC class II

Table 1. Combined immunodeficiencies

Designation	Serum Ig	Circulating B cells	Circulating T cells	Presumed pathogenesis	Inheritance	Associated features
1. T-B+ SCID						
(a) X-linked (γc deficiency)	Decreased	Normal or increased	Markedly decreased	Mutations in γ chain of IL2,4,7,9,15 receptors	XL	
(b) Autosomal recessive (Jak3 deficiency)	Decreased	Normal or increased	Markedly decreased	Mutation in Jak3	AR	
2. T-B-SCID						
(a) RAG 1/2 deficiency	Decreased	Markedly decreased	Markedly decreased	Mutation in RAG1/2 genes	AR	
(b) Adenosine deaminase (ADA) deficiency	Decreased	Progressive decrease	Progressive decrease	T-cell and B-cell defects from toxic metabolites (e.g. dATP, S-adenosyl homocysteine) due to enzyme deficiency	AR	
(c) Reticular dysgenesis	Decreased	Markedly decreased	Markedly decreased	Defective maturation of T and B cells and myeloid cells (stem cell defect)	AR	Granulocytopenia Thrombocytopenia
3. X-linked hyper IgM syndrome	IgM & IgD increased or normal; other isotypes decreased	IgM & IgD bearing cells present others absent	Normal	Mutations in CD40 ligand gene	XL	Neutropenia Thrombocytopenia Haemolytic anaemia Gastrointestinal & liver involvement
4. Purine nucleoside phosphorylase (PNP) deficiency	Normal or decreased	Normal	Progressive decrease	T-cell defect from toxic metabolites (e.g. dGTP) due to enzyme deficiency	AR	Autoimune haemolytic anaemia: neurological symptoms
5. MHC class II deficiency	Normal or decreased	Normal	Normal, decreased CD4 numbers	Mutation in transcription factors (CIITA or RFX-5 genes) for MHC class II molecules	AR	
6. CD3 γ or CD3ε deficiency	Normal	Normal	Normal	Defective transcription of CD3γ or CD3ε chain	AR	
7. ZAP-70 deficiency	Normal	Normal	Decreased CD8, normal CD4	Mutations in Zap-70 kinase gene	AR	
8. TAP-2 deficiency	Normal	Normal	Decreased CD8, normal CD4	Mutations in TAP-2 gene	AR	MHC class I deficiency

molecules and CD18 on fetal blood cells can identify MHC Class II deficiency and the leukocyte adhesion defect 1.

9 PRIMARY SPECIFIC IMMUNODEFICIENCY

9.1 Introduction

Nomenclature and characteristics of currently recognized Primary Immunodeficiency Diseases are given in Tables 1, 2, 3 and 8. The columns provide the points below.

9.1.1 Designated nomenclature. Nomenclature that defines the presumed cause or the most characteristic expression of the disease is generally used. Eponyms have been avoided because original descriptions from which they were derived preceded modern immunological techniques and, as a result, may be misleading. Precise nomenclature and standardized diagnostic criteria are crucial for case documentation, comparison and compilation of registries.

9.1.2 Serum immunoglobulin levels. Defective antibody formation is the most common abnormality in the majority of Primary Immunodeficiency Diseases. It is generally reflected by decreased total serum Ig. Thus serum antibody and serum Ig concentrations are combined under a single heading.

9.1.3 Circulating B and T lymphocytes. Enumeration and characterization of circulating lymphocytes is essential for the diagnosis. Methods for T- and B-lymphocyte enumeration and differentiation markers are given in Sections 6.2.3 and 6.3.2 and for function analysis in Sections 6.3.1 and 6.3.3. Skin tests for delayed hypersensitivity (cell mediated immunity – CMI) generally reflect

Table 2. Predominantly antibody deficiencies

Associated designation	Serum Ig	Circulating B cells	Presumed pathogenesis	Inheritance	Associated features
1. X-linked agammaglobulinaemia	All isotypes decreased	Profoundly decreased	Mutations in *btk* gene	XL	-
2. Non X-linked hyper IgM syndrome	IgM and IgD increased or normal other isotopes decreased	IgM and IgD bearing cells present others absent	Uknown	?	Neutropenia Thrombocytopenia Haemolytic anaemia Gastrointestinal and liver involvement
3. Ig heavy-chain gene deletions	IgG1 or IgG2, IgG4 absent and in some cases IgE and IgA2 absent	Normal or decreased	Chromosomal deletion at 14q32	AR	-
4. κ Chain deficiency mutations at AR	Ig(K) decreased: antibody response normal or decreased	Normal or decreased κ-bearing cells	Point mutations at chromosome 2p11 in some patients	AR	-
5. Selective deficiency of IgG subclasses with or without IgA deficiency	Decrease in one or more IgG isotypes	Normal or immature	Defects of isotype differentiation	Unknown	-
6. Antibody deficiency with normal Igs	Normal	Normal	Unknown	Unknown	-
7. Common variable immunodeficiency	Various decreases of multiple isotypes	Normal or decreased	Variable; undetermined	Variable	See text Section 9.3.6
8. IgA deficiency	IgA1 and IgA2 decreased	Normal or decreased sIgA+	Failure of terminal differentiation in IgA+B cells	Variable	Autoimmune and allergic disorders
9. Transient hypogammaglobulinaemia of infancy	IgG and IgA decreased	Normal	Differentiation defect: delayed maturation of helper function	Unknown	Frequent in families with other IDs
10. Autosomal recessive agammaglobulinaemia	All isotopes decreased	Profoundly decreased	Intrinsic defect pre-B to B-cell differentiation	AR	-

T-lymphocyte numbers and *in vitro* functional assays, they are thus omitted from the listed characteristics.

9.1.4 Presumed pathogenesis. Many Primary Immunodeficiency Diseases result from impeded B or T lymphocyte development and differentiation. The normal ontogeny is described in Section 2 (Cellular basis of the immune response), and is schematically shown in Fig. 1. The probable location of the arrest in development or differentiation is indicated where possible. In the few instances where the defect can be more precisely identified, greater details are given.

9.1.5 Inheritance. Many of the Primary Immunodeficiency Diseases are inherited. The inheritance of those that have been well defined is noted. Approximate chromosomal gene map location is given in Table 6.

9.1.6 Associated features. Commonly associated, characteristic and often diagnostic non-immunological features for some of the Primary Immunodeficiency Diseases are listed. Additional conditions that have been associated with Immunodeficiency are described in Section 10 (Immunodeficiency associated with or secondary to other diseases).

9.2 Combined immunodeficiencies (CID)

This group of diseases (Table 1) is characterized clinically and immunologically by defects in both T and B lymphocytes. Criteria for diagnosis generally include presentation in infancy with severe, potentially lethal infections, profound abnormalities of CMI and antibody deficiency, and lymphopenia, particularly of T lymphocytes. The clinical presentation usually includes failure to thrive and unusually persistent infections with low virulence opportunistic organisms (for example, Candida, *Pneumocystis carinii*, cytomegalovirus). These findings require differentiation from infants with AIDS. HIV studies should include viral isolation or PCR studies for viral genome. SCID is further distinguished on the basis of pathogenesis where known (e.g. enzyme defects), mode of inheritance and level of faulty differentiation.

Table 3. Other well-defined immunodeficiency syndromes

Designation	Serum Ig and antibodies	Circulating B cells	Circulating T cells	Genetic defect	Inheritance	Associated features
1. Wiskott–Aldrich syndrome	Decreased IgM: antibody to polysaccharides particularly decreased; often increased IgA and IgE	Normal	Progressive decrease	Mutations in WASp gene; cytoskeletal defect affecting haematopoietic stem cell derivatives	XL	Thrombocytopenia; small defective platelets; eczema; lymphomas; autoimmune disease
2. Ataxia-telangiectasia	Often decreased IgA, IgE and IgG subclasses; increased IgM monomers; antibodies variably decreased	Normal	Decreased	Mutation in A-T gene (*ATM*); disorder of cell cycle checkpoint pathway leading to chromosomal instability	AR	Ataxia; telangiectasia; increased alpha fetoprotein; lymphoreticular and other malignancies; increased X-ray sensitivity
3. DiGeorge anomaly	Normal or decreased	Normal	Decreased or normal	Contiguous gene defect in 90% affecting thymic development	De novo defect or AD	Hypoparathyroidism; conotruncal malformation; abnormal facies; partial monosomy of 22q11-pter or 10p in some patients

Table 4. Complement deficiencies

Deficiency	Inheritance	Chromosomal location	Chromosomal symptom
C1q	AR	1	SLE-like syndrome, rheumatoid disease, infection
C1r*	AR	12	SLE-like syndrome, rheumatoid disease, infection
C4	AR	6	SLE-like syndrome, rheumatoid disease, infection
C2**	AR	6	SLE-like syndrome, vasculitis, polymyositis
C3	AR	19	Recurrent pyogenic infections
C5	AR	9	Neisserial infection, SLE
C6	AR	5	Neisserial infection, SLE
C7	AR	5	Neisserial infection, SLE, vasculitis
C8α***	AR	1	Neisserial infection, SLE
C8β	AR	1	Neisserial infection, SLE
C9	AR	5	Neisserial infection
C1 inhibitor	AD	11	Hereditary angioedema
Factor I	AR	4	Recurrent pyogenic infections
Factor H	AR	1	Recurrent pyogenic infections
Factor D	AR	?	Neisserial infection
Properdin	XL	X	Neisserial infection

* C1r deficiency in most cases is associated with C1s deficiency. The gene for C1s also maps to chromosome 12 p ter.

** C2 deficiency is in linkage disequilibrium with HLA-A25, B18 and -DR2 and complotype, SO42 [slow variant of Factor B, absent C2, type 4 C4A, type 2 C4B].

*** C8α deficiency is always associated with C8γ deficiency. The gene encoding C8γ maps to chromosome 9 and is normal but C8γ covalently binds to C8α.

9.2.1 Severe combined Immunodeficiency (SCID). Patients with SCID can be divided into two large groups: (i) those who lack both T and B lymphocytes (T-B-SCID), and (ii) those who have normal to increased B cells, and lack of T lymphocytes (T- B+ SCID). X-linked, T- B+ SCID is the most common form of SCID, and is due to mutations in the γc chain shared by the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15. Autosomal recessive, T- B+ SCID is due to mutations of the intracellular kinase, Jak3, that binds to γc. Some patients with SCID have symptoms similar to graft versus host disease (GVH) in the neonatal period. This has been termed 'Omenn's Syndrome'; the disease is not, however, due to engraftment of maternal cells. The genetic and molecular bases of several forms of SCID have been determined and are listed in Table 8 and described in Section 9.4. Bone marrow transplantation is the treatment of choice for SCID, with excellent results.

9.2.1.1 RAG-1/-2 deficiency. Many patients with T-B- SCID have mutations in RAG-1 or RAG-2 genes. These infants have very few B or T cells but may have normal or increased numbers of NK cells. The defects are inherited as an autosomal recessive.

9.2.1.2 Adenosine deaminase (ADA) deficiency. There is a group of distinctive patients whose SCID results from defects in the enzyme ADA. This group of phenotypically similar genetic defects include various mutations within the gene encoding ADA on chromosome 20q13-ter. In the absence of ADA, toxic metabolites of the purine pathway (dATP) and the methylation pathway (S-adenosyl homocysteine) accumulate within the cell and impair proliferation; as a result both T- and B-lymphocyte functions are defective. Inheritance of the defect is autosomal recessive. Rare patients with certain mutations in *ADA* that result in a mild phenotype may have late onset of ID.

9.2.1.3 Reticular dysgenesis. This rare hereditary autosomal recessive disease is generally lethal shortly after birth. It results from

failure in the maturation of both lymphoid and myeloid precursors, and is characterized not only by striking lymphopenia, but also by severe granulocytopenia and thrombocytopenia, and overwhelming infections with early death. Engraftment of maternal T cells in SCID infants may occasionally mimic reticular dysgenesis.

9.2.2 Immunoglobulin deficiency with increased IgM (the hyper-IgM syndrome). This syndrome apparently represents a group of distinct entities with similar clinical (and phenotypic) expression. 70% of the cases are X-linked in inheritance; others have been autosomal recessive. Diagnostic criteria include impeded antibody formation. Patients may have an intact IgM antibody response. There is no switch to IgG antibody formation. Thus serum IgM (and sometimes IgD) levels are elevated while IgG and IgA levels are diminished. Circulating B lymphocytes bear only IgM and IgD. The defect is a failure of isotype switch but there is no defect in the switch regions of B lymphocytes. Most patients have recurrent or persistent neutropenia and thrombocytopenia. Defects in CMI have been noted in some patients.

In the X-linked form, the genetic defect has been identified in mutation of the gene for the CD40 ligand, which is expressed on activated T lymphocytes. The interaction of the CD40 ligand with CD40 on B lymphocytes is requisite for productive isotype switching. The gene for the CD40 ligand maps to Xq26, where the hyper-IgM syndrome had previously been mapped. The CD40 ligand is a type 2 glycoprotein that belongs to the same gene family as tumour necrosis factor. In most cases no CD40 ligand is expressed on the T cells of these patients. In others a mutant non-functional protein is expressed and these patients may have a less severe phenotype.

Table 5. Congenital defects of phagocytic number and/or function

Disease	Affected cells	Functional defects	Inheritance	Features
Severe congenital neutropenia	N	-	AR	Subgroup with G-CSF-R mutation and MDS/AML
Cyclic neutropenia	Mainly N	-	AR	Oscillations of reticulocytes, platelets and other leukocytes
Leukocyte adhesion defect 1 [deficiency of beta chain (CD18) of LFA-1, Mac 1, p150,95]	N + M + L + NK	Chemotaxis, adherence, endocytosis	AR	Delayed cord separation, chronic skin ulcers, periodontitis, leukocytosis, defective T+NK-cell cytotoxicity
Leukocyte adhesion defect 2 (failure to convert GDP mannose to fucose)	Mainly N + M	Chemotaxis, rolling	AR	Delayed wound healing, chronic skin ulcers, periodontitis, mental retardation, leukocytosis, Bombay blood group
Chediak–Higashi syndrome	Mainly N+M+NK	Chemotaxis	AR	Oculo-cutaneous albinism, giant granules of all nucleated cells, terminal, haemophagocytic syndrome
Specific granule deficiency	N	Chemotaxis	AR	N with bi-lobed nuclei
Schwachman syndrome	N	Chemotaxis	AR	Anaemia, thrombocytopenia, pancreatic insufficiency, chondro-dysplasia, hypogammaglobulinaemia
Chronic granulomatous disease (a) X-linked CGD (deficiency of 91kD chain of cytochrome b)	N + M	Killing (faulty production of superoxide metabolites)	XL	McLeod phenotype*
(b) Autosomal recessive (deficiencies of 22 kD chain of cytochrome b or of P47 or P67 cytosol factors)	N+M	Killing — as above	AR	-
Neutrophil G6PD deficiency	N+M	Killing	XL	Anaemia
Myeloperoxidase deficiency	N	Killing	AR	-
IFN-γ receptor deficiency	N+M+L+NK	Killing	AR	Extreme susceptibility to mycobacteria

N=neutrophils; M=monocytes/macrophages; L=lymphocytes; NK= natural killer cells
*Some patients have deletions in the short arm of the X chromosome; in these patients additional features including McLeod phenotype, retinitis pigmentosa and Duchenne muscular dystrophy may be found.

Table 6. Chromosome map location of IDs listed in Tables*

1. X-linked severe combined immunodeficiency	Xq13.1-13.3
2. X-linked agammaglobulinaemia	Xq21.3-22
3. X-linked immunodeficiency with increased IgM	Xq26-27
4. Wiskott–Aldrich syndrome	Xp11.22-11.3
5. X-linked chronic granulomatous disease	Xp21.1
6. X-linked lymphoproliferative syndrome	Xq26
7. Adenosine deaminase deficiency	20q13-ter
8. Purine nucleoside phosphorylase deficiency	14q13.1
9. ZAP-70 deficiency	2q12
10. Jak3 deficiency	19p13.1
11. RAG-1/RAG-2	11p12-13
12. Kappa chain deficiency	2p11
13. Ig heavy chain deletion	14q32.3
14. Ataxia-telangiectasia	11q23.1
15. Autosomal recessive chronic granulomatous disease	
p22 phox	16q24
p47 phox	7q11.23
p67 phox	1q25
16. Leukocyte adhesion deficiency I	21q22.3
17. IFN-γR α chain deficiency	6q16-21
18. Chediak–Higashi syndrome	1q4.3
19. TAP-2	6p21.3
20. CIITA	16p13.1-2
21. RFX5	1q21

* The complement gene map locations (and hence the deficiencies thereof) are given in Table 5.

9.2.3 Purine nucleoside phosphorylase (PNP) deficiency. This autosomal recessive disease results from defects in the gene encoding the enzyme PNP located on chromosome 14. In the absence of PNP, toxic metabolites, in this case dGTP, accumulate within the cell and impair proliferation. T lymphocytes are particularly sensitive to the accumulation of dGTP and they are affected to a greater degree than B lymphocytes. There are thus immunological differences between ADA and PNP deficiency.

9.2.4 MHC class II deficiency. The disease is due to a defect in proteins that promote transcription of class II molecules. The disease is heterogeneous and four complementation groups are presently known. Complementation group A results from mutations in the gene encoding class II transcription activation (CIITA). Complementation group C results from mutation in the genes for the heterodimer RFX5, whereas mutations of p36 (that binds to RFX5) account for complementation group D. In the absence of class II MHC molecules, cognitive functions, particularly those involving CD4+ T lymphocytes, are impaired. Circulating lymphocyte numbers are normal, but CD4+T cells are decreased. Antibody synthesis and serum immunoglobulins are decreased and CMI is defective. Several of these children have been recipients of successful bone marrow transplants. An unusual phenotype with residual expression of HLA-DRα and β chains is associated with a normal number of circulating CD4+ lymphocytes, and a more benign clinical course.

9.2.5 CD3 deficiency. The phenotype of CD3 deficiency may be variable, even within a family, due to variable expression of CD3 on the T-cell membrane. Deficiencies or abnormalities of CD3 γ and ε have been described.

9.2.6 ZAP-70 deficiency. This rare deficiency is inherited as an autosomal recessive trait and is due to mutations in the gene for ZAP-70 (see Fig. 4), a tyrosine kinase involved in TCR signalling. CD4+ T cells are present in normal or elevated numbers but are not functional. Some of these children have been recipients of successful bone marrow transplants.

9.2.7 TAP 2 peptide transporter deficiency. In one family, an immunodeficiency characterized by reduced HLA class I molecules expression, low CD8 T-cell counts and defective cytotoxicities has been found in association with mutation in the gene encoding the peptide transporter chain TAP2. The latter is necessary for the transport of peptides into the endoplasmic reticulum for binding to the groove of MHC class I molecules.

9.3 Predominantly antibody defects

The defect in several of the Primary Immunodeficiency Diseases is restricted to antibody formation, either from impeded B-lymphocyte development or failure of effective B-lymphocyte responses to T-lymphocyte signals. This group of diseases, summarized in Table 2, presents clinically with recurrent pyogenic infections.

Table 7. Prenatal diagnosis

Diseases	Informative restriction fragment length polymorphisms	Findings in fetal cord blood or amnion cells
X-linked agammaglobulinaemia	-	Absence of B cells
X-linked severe combined immunodeficiency	-	Absence of T cells
Autosomal recessive severe combined immunodeficiency	-	Absence of T cells (and B cells)
Wiskott–Aldrich syndrome	+	'Bald' lymphocytes by scanning EM
Ataxia-telangiectasia	+	Radiosensitivity
MHC class II deficiency	-	Absence of MHC class II molecules on cell membranes
Leukocyte adhesion deficiency	(*)	Absence of CD18 on phagocytes
X-linked chronic granulomatous disease	+	Abnormal oxygen radical production
Autosomal recessive chronic granulomatous disease	(*)	Abnormal oxygen radical production
Adenosine deaminase deficiency	+	Decreased ADA in red blood cells
Purine nucleoside phosphorylase deficiency	-	Decreased PNP in red blood cells

*Potentially possible, but not yet well established.

Table 8. Other primary immunodeficiency diseases

Primary CD4 deficiency
Primary CD7 deficiency
IL-2 deficiency
Multiple cytokine deficiency
Signal transduction deficiency ± myopathy
Calcium flux deficiency with myopathy

9.3.1 X-linked agammaglobulinaemia. This is the prototypic antibody deficiency. Affected males present in infancy or early childhood with recurrent pyogenic infections. The tonsils are small and lymph nodes are usually not palpable. Criteria for diagnosis include profound inability to make antibody and resultant low concentrations of all immunoglobulin isotypes. There is a decrease in circulating B lymphocytes (usually less than 5/1000 lymphocytes); plasma cells and germinal centres are absent. The number and function of T lymphocytes (including cell-mediated immunity) are unaffected. Pre-B cells are normally found in the bone marrow. The gene defect has been localized to the long arm of the X chromosome (Xq21.3-22). XLA is due to mutations in a cytoplasmic tyrosine kinase designated *btk* or Bruton's agammaglobulinaemia tyrosine kinase. It consists of an N-terminal pleckstrin homology domain (PH), followed by a Tec (TH) domain, a Src homology 3, i.e. a SH3 domain, a SH2 domain and a C-terminal SH1 or tyrosine kinase domain. Mutations in all five domains of *btk* have been found in XLA. The xid mutation in mice is due to a missense mutation in which an arginine at residue 28 in the PH domain is converted to a cysteine. In female carriers of XLA the defective chromosome is preferentially lyonized during B-lymphocyte proliferation. The clinical phenotype may be very variable, even within the same family. Since the identification of the gene defect, it has been appreciated that the clinical phenotype is broader than originally conceived and all young males with a predominant antibody defect should be examined for mutations in *btk*.

9.3.1.1 X-linked hypogammaglobulinaemia with growth hormone deficiency. X-linked agammaglobulinaemia associated with primary growth hormone deficiency has been reported in a family in which there was no abnormality of *btk* expression. The genetic defect causing this disorder is yet unknown.

9.3.2 Non X-linked hyper IgM syndrome. See 9.2.2.

9.3.3 Ig heavy chain deletion. Deletions and duplications in chromosome 14q32 of the heavy chain constant region genes occur in 5–10 % of the Caucasian population and are probably common in all ethnic groups. Individuals who are homozygous for such deletions lack the relevant isotypes and subclasses. Heterozygotes often show slightly diminished levels of the affected subclasses. Most such families were found during the screening of entirely well, normal blood donors who had no history of recurrent infections. A few individuals homozygous for these defects have presented with recurrent pyogenic infections.

Deletions and point mutations affecting the expression of the surface bound μ heavy chain have recently been found not only to cause a lack of IgM but also to lead to a defect in B lymphocyte development resulting in agammaglobulinaemia. It is unlikely that this novel form of immunodeficiency can account for a proportion of female patients with a clinical picture resembling XLA.

9.3.4 κ chain deficiency. Two families have been described whose immunoglobulin chains have λ light chains only. No κ chains were found. Antibody formation was variable; circulating B lymphocytes were normal except that they did not carry κ light chain. Point mutations in the κ chain gene located at chromosome 2p11 were reported in one family.

9.3.5 Selective IgG subclass deficiency. Criteria for diagnosis should include normal total serum IgG levels with sub-normal levels of one or more IgG subclasses. It is difficult to be certain of normal subclass levels. As noted in Section 6.2.1, the assays for subclasses are not well standardized; age-related and population-related norms are not always available; genetic variation exists among individuals in different ethnic groups. Since IgG1 is the predominant serum IgG subclass, deficiency of IgG1 cannot generally occur without a decrease in total serum IgG, in which instance the defect should be considered as 'Common Variable Immunodeficiency'. IgA levels are frequently, but not invariably, decreased in patients with IgG2 deficiency. Low levels of IgG3 are the most common IgG subclass abnormality reported in adults, whereas low levels of IgG2 are more common in children, particularly in association with poor responses to polysaccharide antigens. IgG4 levels vary widely in normal persons, and many entirely normal persons have no demonstrable IgG4 by standard techniques; selective deficiency of IgG4 alone is difficult to interpret. IgG2 deficiency, which is often associated with low or undetectable IgG4 levels and an inability to respond to polysaccharide antigens, may be confused with 'Antibody Deficiency with Normal Immunoglobulins'.

Selective immune globulin subclass deficiencies were shown to be present in healthy blood donors without undue susceptibility to infection. Deletions of immune globulin heavy-chain genes could be detected in these individuals.

9.3.6 Selective antibody deficiency with normal immunoglobulins. It has been known for decades that some individuals selectively fail to respond to certain antigens. The characteristic defect is failure to respond to polysaccharide antigens. While most such persons are normal, some have recurrent sino-pulmonary infections. Criteria for diagnosis should include demonstrated failure to response to specific antigens, a normal response to other antigens and normal total serum IgG and IgM levels. In some of these persons diminished serum IgG2 levels have been found. This appears to be an associative not causative relationship; IgG2 levels are not predictive of antibody responses. Antibody responses to polysaccharide antigens are often found to be diminished in persons with sickle cell anaemia, asplenia (see Section 10), the Wiskott–Aldrich syndrome (Section 9.5.1) and the DiGeorge syndrome. In uncontrolled case studies, patients non-responsive to polysaccharide antigens with normal immunoglobulins and chronic sinopulmonary disease benefited from IgG replacement. Non-responders to polysaccharide antigens produce antibody well with conjugate vaccines. Some individuals who are not responsive to hepatitis vaccine and other protein antigens may fall into this category.

9.3.7 Common variable immunodeficiency (CVID). The term 'Common Variable Immunodeficiency' (CVID) is used to describe an incompletely defined syndrome characterized by defective antibody formation. The diagnosis is otherwise based on exclusion of other known causes of humoral immune defects. The term 'acquired immunodeficiency' (AIDS) should be reserved for patients in whom the diagnosis of HIV infection has been established.

Perhaps because it has not yet been differentiated into its many probably distinct component syndromes, CVID is one of the most frequent of the primary specific immunodeficiency diseases: the incidence has been estimated at 1:10,000 to 1: 50,000. Affecting males and females equally, the usual age of presentation is the second or third decade of life.

In common with all primary immunodeficiencies affecting humoral immunity, the clinical presentation of CVID is generally that of recurrent pyogenic sinopulmonary infections. Early diagnosis is important; some patients are only discovered when they have significant chronic lung disease, including bronchiectasis.

As with XLA, some patients develop unusual enteroviral infections with a chronic meningo-encephalitis, and other manifestations including a dermatomyositis-like syndrome. Patients with CVID are also highly prone to gastrointestinal infections caused by *Giardia lamblia* and *Campylobacter jejuni*.

There is an unusually high incidence of lymphoreticular and gastrointestinal malignancies in CVID. Lymphoproliferative disorders are often apparent from physical examination, where in contrast to XLA, up to a third of CVID patients have splenomegaly and/or diffuse lymphadenopathy. The lymph nodes show a striking reactive follicular hyperplasia. Non-caseating granulomas resembling sarcoidosis and striking non-malignant lymphoproliferation occur. The gastrointestinal tract may also be involved in this process with a characteristic nodular lymphoid hyperplasia. Malabsorption with weight loss and diarrhoea and associated changes such as hypoalbuminaemia, vitamin deficiencies and other findings resembling coeliac sprue are seen. Chronic inflammatory bowel diseases occur with increased frequency. Patients with CVID are prone to a variety of other autoimmune disorders (e.g. pernicious anaemia, haemolytic anaemia, thrombocytopenia and neutropenia).

The *sine qua non* for the diagnosis of CVID is defective antibody formation. These are usually accompanied by decreased serum IgG and IgA levels and generally but not invariably decreased serum IgM. Because CVID is a diagnosis of exclusion, those patients with elevated or high normal levels of serum IgM should be evaluated for the Hyper-IgM syndrome (see Section 9.2.2). Male patients with very low or undemonstrable IgG, especially if they have markedly diminished numbers of circulating B cells, should be evaluated for XLA (see Section 9.3.1). In some patients cell-mediated immunity (CMI) may be impaired with diminished T-cell function, and absent DTH; the immunodeficiency under these circumstances involves both cellular and humoral immunity and the disease could be considered as a 'Combined Immunodeficiency' although the clinical expression is primarily defective antibody production.

As noted in Section 9.3.8, IgA deficiency is common in the general population. In CVID, IgA levels are undetectable or markedly below the normal range in almost all patients. Family members may also have an unusually high incidence of IgA deficiency. In addition families of patients with CVID have an increased incidence of autoimmune disorders, auto antibodies (including anti-lymphocyte antibodies) and malignancies, suggesting a wide expression of immune dysregulation. As would be expected in a heterogeneous group of undifferentiated diseases, various inheritance patterns for CVID (autosomal recessive, autosomal dominant, X-linked) have been noted. Sporadic cases with no obvious inheritance pattern are, however, the most common. In multiplex families containing several persons with CVID and IgA deficiency involved individuals often inherit characteristic MHC alleles.

Many studies to identify the immunological defect(s) have been published. None to date has provided patterns sufficiently consistent for classification. There is no convincing evidence for any intrinsic B-cell defect of immunoglobulin genes, synthesis or secretion. While B cells (defined as CD19+) may be reduced in number, with appropriate stimulation they produce and secrete immunoglobulins.

CVID patients commonly have reduced CD4/CD8 ratios, with a reduction in CD4+CD45RA+ ('unprimed') T cells and this suggests that there has been activation of T cells. The reported increased levels of IL-4 and IL-6, soluble CD8, CD25, β2-microglobulin, HLA-DR, LAF-3 and ICAM-1 are probably secondary to infection.

About 60% of CVID patients have diminished proliferative responses to T-cell receptor stimulation, and decreased induction of gene expression for IL-2, IL-4, IL-5 and IFNγ. There is no evident abnormality of the T-cell receptors: T-cell receptor gene analyses indicates normal heterogeneity of gene rearrangements. There is decreased IL-2 production after T-cell receptor stimulation, which is correlated with diminished CD40 ligand expression. The abnormality appears to reside in CD4+ T cells and can be overcome by stimulating T cells with PMA and ionomycin – an alternative T-cell activation pathway (see Section 5). This is consistent with defective signal transduction, which could explain the diminished humoral immunity.

9.3.8 IgA deficiency. About 1 in 700 Caucasians (in contrast to 1:18,500 Japanese individuals) have no demonstrable serum IgA. Many of these individuals have no apparent disease. Some persons with recurrent sinopulmonary infections have been reported with entirely normal serum IgM and IgG levels, but absent or extremely reduced serum IgA levels. Whether the IgA deficiency or some other factors are involved in their illnesses is not clear. IgA deficiency is however more frequent in patients with chronic lung disease than in a normal age-matched population. Furthermore, IgA deficiency is more frequent in patients with autoimmune disease than in the normal healthy population. The defect is presumed to result from impaired switching or a maturational failure of IgA producing lymphocytes. Autosomal recessive or dominant inheritance has been shown in some families. Fixed haplotypes of MHC genes are frequently associated with CVID and IgA deficiency and both disorders may reflect a continuum of the same underlying pathogenetic mechanism.

9.3.9 Transient hypogammaglobulinaemia of infancy. Maternal IgG is actively transferred to the fetus throughout pregnancy. The serum IgG level of full-term infants is equal to or slightly greater than that of the mother. Maternal IgG in the infant disappears after birth with a half-life of 25–30 days and the infants own Ig production is initiated, starting with IgM and followed by IgG and then IgA. The time of initiation and the rate of production of Ig by infants varies considerably. During the first 3–12 months of life in premature infants (where the transfer of maternal IgG is often limited) and in some full-term infants (particularly in families with immunodeficiency), the nadir of serum Ig concentration may be very low– within an 'immunodeficient' range. The initiation of antibody production may be delayed for as long as 36 months and ultimately is manifested by increased levels of serum IgG. Antibody production by the infants themselves can usually be documented by serial measurement of serum IgG levels and of antibody responses to vaccine antigens.

9.4 Predominantly T-cell defects

In addition to the ID diseases listed in Tables 1, 2, and 3, other primary defects in the immune system where the genetics and pathogenesis of the ID are not yet completely understood have been described in isolated cases and are listed in Table 8.

9.4.1 Primary CD4 T-cell deficiency. Profound, persistent decrease in circulating CD4+ T lymphocytes, with defective CMI, has been documented in patients, not infected by HIV, who present with opportunistic infections, such as cryptococcal meningitis and oral candidiasis. Immunoglobulin levels may be normal or slightly decreased. The pathogenesis and genetics of this abnormality are not yet known. When such patients are identified CD4 enumeration should be carried out in other family members.

9.4.2 Primary CD7 T-cell deficiency. Children with SCID with CD7+ T-cell deficiency have been described. No genetic transmission of the defect could be ascertained.

9.4.3 IL-2 deficiency. Children with SCID and normal circulating T-cell numbers was found to be unable to transcribe the IL-2 gene. The inheritance of the defect could not be determined.

9.4.4 Multiple cytokine defect. Children with SCID who were deficient in IL-2, IL-4, IL-5 and interferon-γ have been described. T cells lacked the nuclear factor of activated T cells (NFAT) promoter. The genetics of the defect are not yet known.

9.4.5 Signal transduction defect. A few children with SCID or CID fail to show normal calcium flux and diacylglycerol generation after antigenic stimulation of their T cells. The defect can be circumvented by stimulation with PMA or aluminum tetrafluoride (AlF_4). The genetics of this condition are not known and the precise defect(s) is not well characterized.

9.4.6 Calcium flux defect. Defective T-cell activation was found associated with an exquisite abnormality in calcium influx from the extracellular milieu in three patients with combined immunodeficiencies. Although the Ca++ flux defect is detected in all types of cells, phenotype is restricted to a T-cell deficiency. Molecular identification of the defect is still awaited.

9.5 Immunodeficiencies associated with other major defects

There are a variety of diseases in which immunodeficiency is an important but not exclusive component. Included in this section (see Table 3) are those diseases where immunodeficiency is the dominant manifestation in syndromes with other defects.

9.5.1 Wiskott–Aldrich syndrome. This X-linked disease presents in infancy or early childhood. Clinical manifestations include eczema, recurrent, often unusual or unresponsive infections, and thrombocytopenia. The platelets are small. Surface sialoglycoproteins, CD43 and gpIb, and other sialoglycoproteins are unstable in the membranes of leukocytes and platelets. The lymphocytes have a characteristic 'bald' appearance on scanning electron microscopy. The cytoskeleton in the T cells and platelets is abnormal and the actin in these cells does not bundle normally. The proliferative response of the T cells to anti-CD3 is absent or greatly diminished. Serum immunoglobulins may at first be normal, but a progressive decrease of IgM develops. Antibody production, especially but not exclusively to polysaccharide antigens, is impaired. Progressive lymphopenia, most marked in the T lymphocyte series with resulting defective CMI, develops. Autoimmune diseases including severe vasculitis and glomerulonephritis may be present. Death occurs in late childhood or in the second to fourth decades of life, often from lymphoma. The defective gene is on the short arm of the X chromosome at Xp11.22 and is selected against during differentiation of all blood cells; thus carrier detection is possible. The gene has been cloned and encodes a protein of 502 amino acids, which has been called the Wiskott–Aldrich syndrome protein (WASp). Its function is not well understood but appear to be involved in signal transduction for cytoskeletal reorganization. Mutations in the WASp gene also cause so-called X-linked thrombocytopenia.

9.5.2 Ataxia-telangiectasia. This autosomal recessive syndrome is characterized by progressive cerebellar ataxia, the appearance of fine telangiectases, especially on ear lobes and conjunctival sclera, and eventually, in most patients, recurrent sinopulmonary infections. Raised levels of serum alpha fetoprotein are present in 95% of patients. Immunodeficiency, while not invariably demonstrable in the early life of affected persons, develops in at least 70% of cases. There is no consistent immunological pattern; no single abnormality has been found to exist in all patients. Serum Ig is decreased in varying patterns: IgG2, IgG4, IgA and IgE are commonly low or absent. Antibody responses to polysaccharide and protein antigens may be reduced. The numbers and function of circulating T lymphocytes, including DCH, are generally diminished. There is an increased incidence of autoantibodies.

Cells from patients with ataxia-telangiectasia have a disorder of their cell cycle checkpoint pathway that results in an extreme hypersensitivity to ionizing radiation. Lymphocytes show frequent chromosomal breaks, inversions and translocations involving sites of the T-cell receptor genes and immunoglobulin gene complexes on chromosomes 7 and 14. In fibroblasts chromosomal breaks, inversions and translocations are random. A-T patients and their parents have a strikingly increased susceptibility to malignancies. Breast cancer in female A-T carriers is reported to be increased. The overall risk of cancer in heterozygotes generally is probably also increased. Death in patients usually occurs in early adult life after years of increasing disability from pulmonary disease or (often lymphoreticular) malignancy. A-T cells fail to upregulate p53 expression following DNA damage by irradiation, indicating that the A-T protein functions upstream from p53 and plays a major role in sensing, but not in repairing, double-stranded DNA breaks.

The gene *ATM* (for A-T *mutated*) was isolated in 1995 by positional cloning. More than 150 mutations have been identified; 80% of these result in truncation of the protein. Most of these mutations are unique. Most patients are compound heterozygotes. The gene product has strong homology to phosphoinositide 3-kinase and preliminary results confirm a protein kinase activity. *ATM* knockout mice show defects in T-cell maturation and develop lymphoid tumours similar to those seen in patients, especially malignant thymic lymphomas. The mice die at 2–4 months with some neurological dysfunction but few if any signs of ataxia.

The Nijmegen Breakage Syndrome (NBS) overlaps with that of A-T; patients are radiosensitive, immunodeficient, cancer prone and similarly manifest reciprocal translocations involving chromosomes 7 and 14. However, they do not have ataxia, telangiectasia nor telangiectases. They are mentally retarded and microcephalic. Family studies do not link the NBS gene to chromosome 11q23.1, the site of the *ATM*. The A-$T_{Variant}$ syndrome overlaps both classical A-T and NBS, including immunodeficiency and an elevated AFP.

For several syndromes with immunodeficiency and chromosomal instability see Section 10.1.

9.5.3 The DiGeorge anomaly. The DiGeorge anomaly is one of a series of contiguous gene syndromes that affect multiple organs during early embryogenesis. Almost all (80–90%) patients with the

DiGeorge anomaly have deletions (often microdeletions) of 22q11-ter. There are several other syndromes with deletions located to the same area. Because they all involve deletions of 22q11-ter they have been termed 'CATCH 22', an acronym for the involved organs: *c*ardiac abnormalities, *a*bnormal facies, *t*hymic hypoplasia, *c*left palate and *h*ypocalcaemia. This group of syndromes would include the velocardiofacial (Shprintzen) syndrome, the conotruncal anomaly face syndrome, Cayler syndrome and some patients with Opitz GBBB syndrome. Additional cases of the DiGeorge anomaly may derive from 10p deletions, from the fetal alcohol syndrome, retinoic embryopathy or maternal diabetes. The characteristic pathological manifestations include multiple anomalies of the third and fourth branchial arch derivatives; Type 1 truncus arteriosus, dysmorphic facies with micrognathia, thymic and parathyroid hypoplasia or aplasia. Clinically, neonatal tetany and/or cardiac failure are the presenting manifestations in most affected infants. The facial features then arouse suspicion as to the diagnosis.

Infections are usually not a presenting manifestation. Even though the thymus is frequently involved, only about 20% of those with the anomaly have decreased numbers and function of T lymphocytes. At autopsy the thymus is small, atrophic often containing normal appearing ectopic lobes. Surviving infants over time may naturally acquire functional T cells and the immunodeficiency becomes corrected. It is difficult therefore to assess the value of the various treatment regimens that have been attempted.

9.6 Associated conditions

In addition to the infections of the respiratory and gastrointestinal tracts noted previously, patients with Primary Specific Immunodeficiency are particularly prone to several other conditions.

9.6.1 Malignancies. Age-specific mortality rates for cancer in patients with primary ID exceed by 10–200 times the expected rates for the general population. The majority of cancers are observed in patients with ataxia-telangiectasia and the Wiskott–Aldrich syndrome. The causal relationship is exemplified by the finding that treatment of patients with SCID or Wiskott–Aldrich syndrome by bone marrow transplantation from an MHC matched sibling donor has led to an impressive reduction in this susceptibility to malignancy.

In patients with ataxia-telangiectasia, transpositions, inversions and breaks of chromosomes 7 and 14 at the sites of the T-cell receptor are associated with lymphoreticular malignancies. These patients also develop malignancies of rapidly replicating cells in other organs.

The types of tumour, lymphoreticular malignancies, in all groups of ID are different from those observed in non-selected populations. Some have clear evidence of clonal proliferation, some are associated with Epstein–Barr virus infection. It appears likely that there is an association between ID and oncogenesis. Possible mechanisms include defective immunological surveillance; defective immune response to oncogenic viruses; chronic over stimulation or proliferation of responsive cells to antigens; independent effects of the same common cause (i.e. chromosomal instability in ataxia-telangiectasia). In CVID there is an increase in the incidence of lymphomas and in gastrointestinal malignancies.

Papilloma virus infections also appear to be more frequent in patients with ID with the production of local verrucae, condylomata acuminata and localized, usually genital, intraepithelial neoplasia.

9.6.2 Autoimmunity. Several autoimmune syndromes have been described in association with ID. These include pernicious anaemia, autoimmune haemolytic anaemia, idiopathic thrombocytopenic purpura, systemic lupus erythaematosus, thyroiditis, Sjögren's syndrome, chronic active hepatitis and myasthenia gravis. In addition to autoantibodies against blood cells, autoantibodies to immunoglobulins and various tissue antigens have been observed. Inflammatory bowel disease is a frequent complication of ID; whether the cause is infectious or autoimmune is not always clear.

9.6.3 Atopic allergy. Atopic allergy due to the presence of IgE antibodies to inhaled, ingested or injected proteins affects 15–20% of the general population. It gives rise to clinical symptoms of asthma, rhinitis, eczema, urticaria and anaphylaxis. Patients with primary immunodeficiencies often have symptoms consistent with asthma and/or rhinitis. In many of the cases this is due to chronic non-IgE mediated inflammation. However attempts should be made, particularly in patients who retain the capacity to make a partial antibody response, to rule out an allergic actiology or contribution. This is achieved by performing immediate hypersensitivity skin tests and by measuring IgE antibodies in the serum.

9.6.4 Unusual viral infections. Patients with predominantly antibody defects (particularly with X-linked agammaglobulinaemia) are especially susceptible to chronic viral infections with ECHO or other enteroviruses. This is characteristically a meningo-encephalitis or a dermatomyositis-like syndrome. Such patients may shed virus for years. Virus may be isolated from cerebrospinal fluid and at post mortem from all viscera. Untreated the infection is fatal. High doses and specific intravenous IgG have controlled these infections in some patients.

Several patients with primary ID have been infected with HIV and with hepatitis C virus. Testing for seroconversion to HIV or hepatitis C virus is of little or no use in diagnosis because of the intrinsic inability of patients with ID to make antibody. PCR techniques should be used to ascertain the presence of viral genomic material. It has also been reported that some CVID patients infected with HIV or hepatitis C have shown some recovery of immunoglobulin levels.

9.7 Treatment of specific immunodeficiency

9.7.1 Bone marrow transplantation. Transplantation of bone marrow cells from HLA genotypically identical donors (i.e. matched sibling donors or other HLA identical members of a family) has led to complete immunological reconstitution of most patients with SCID, including those with ADA deficiency, and those with reticular dysgenesis. Bone marrow transplantation has also been successful in the Wiskott–Aldrich syndrome, leukocyte adhesion deficiency, MHC Class II immunodeficiencies, Kostmann's syndrome, chronic granulomatous disease, X-linked hyper IgM and Chediak–Higashi syndrome. Ideally, donor and recipient should be identical at the HLA-A, B, C and DR loci. Unfortunately, three quarters of patients do not have a compatible donor. Great progress has been made in haplo-identical bone-marrow transplantation in recent years. Extensive conditioning of the recipient to prevent rejection and the elimination of T cells from the donor cells to avoid graft-versus-host disease are mandatory. Success with T-cell engraftment has been very encouraging, but it has been difficult to establish B-cell engraftment with haplo-identical bone marrow. Full reconstitution takes longer than engraftment with HLA identical donors.

An increasing number of successful bone marrow transplants are being performed using bone marrow or peripheral blood stem cells from unrelated donors identified in one of the registries of

HLA typing. Attention is being paid to HLA-D match. Stem cells from umbilical cord blood stored in certain centres are being used in an increasing number of cases.

Peripheral blood stem cells from specially pretreated donors after T-cell depletion of the donated cells with or without further purification of the stem cell population is increasingly used in cases of unrelated histocompatible and haplo-identical situations. Intrauterine transplant of parental haematopoietic stem cells has led to successful T cell engraftment in some cases of X-linked SCID.

Acute graft-versus-host (GVH) disease, when it occurs, generally appears 10–14 days after a transplant and is usually manifested by fever, Coombs' test-positive haemolytic anaemia, erythematous, maculopapular skin rash, bloody diarrhoea, hepatosplenomegaly, aregenerative pancytopenia and death. The various means proposed to prevent graft-versus-host disease have included the use of cyclosporin A, alone or together with methotrexate. T-cell depletion of donated bone marrow has also been used. Persistent low-grade GVH reactions, characterized by hepatomegaly, jaundice or skin rashes, can continue for many months and become chronic and severely debilitating.

The establishment of immune competence ('take' of the graft) may be identified by: improvement of clinical status (e.g. weight gain, rapid resolution of moniliasis); appearance of T and B cells in the circulating blood; demonstration of donor cells in the recipient by genetic markers, including enzyme activity in previously deficient patients; increase of immunoglobulin levels (including Ig of donor origin); appearance of humoral antibodies (including those following antigenic stimulation); return of C1q level to normal; and appearance of CMI reactions. Of these, the establishment of chimerism is the most reliable evidence of engraftment. Appropriate tests for mosaicism include sex and other chromosomal studies, molecular analysis at polymorphic loci, HLA and red cell antigens, plasma protein or enzyme allotypes.

Tests of immunological competence should be repeated periodically in successful cases, since subsequent gradual decline of function has been observed in some instances of initially successful engraftment. Children dramatically restored immunologically have also occasionally died of pre-existing pulmonary infections with *Pneumocystis carinii* or other organisms just after immunological capacity has been restored. Prophylactic treatment with sulfamethoxazole-trimethaprim has proven useful in the treatment of these complications. Several deaths from varicella have occurred in successfully transplanted ID patients; such patients should be passively protected with varicella-zoster immune globulin (VZIG) and acyclovir following exposure, if no circulating antibody can be demonstrated. CMV has been successfully treated with gancyclovir and foscarnet and high dose of specific immunoglobulin. Other antiviral agents are being developed and tested at the present time. Many SCID patients who do not have B-cell engraftment (~ 40%) require IgG replacement.

The risk of developing EBV-induced B-cell lymphomas in transplant recipients, particularly of haplo-identical bone marrow donations, has been a difficult and as yet unsolved problem.

9.7.2 Replacement of immunoglobulins. The efficacy of immunoglobulin replacement for antibody deficiency syndromes was well established in the 1950s. It is now accepted that all patients with primary specific immunodeficiency who have significantly diminished serum IgG levels and/or demonstrated defects in antibody production should receive IgG replacement. Preparations suitable for either intramuscular or intravenous use are available for this purpose; the intramuscular preparations should never be given intravenously, but they can be given subcutaneously. Intravenous immunoglobulin replacement is the preferred treatment. Standards for the preparations are the subject of an IUIS/WHO report (Bull. WHO 60(1), 43, 1982). Viral partition and inactivation methods used during the fractionation procedures have been incorporated into the production of immunoglobulin and all manufacturers are required to provide data that validate the viral inactivation methods used. Thus HIV and other retroviruses are effectively excluded by current fractionation procedures, essentially eliminating the risk for transmission of lipid-coated viruses. Clusters of hepatitis C infections have been reported in patients who received certain lots of IVIG.

Experience has shown that replacement therapy with intravenous Ig is life-saving. If replacement is started early, and if appropriate amounts are given with sufficient frequency, the cycle of recurrent infections and progressive lung damage can be arrested. Near normal serum IgG levels can be maintained with ease; general experience suggests that 400–500 mg/kg/month may be sufficient to prevent breakthrough infections. It has been documented that abnormal pulmonary function may improve, even if chronic lung damage is present, if doses of IgG >600 mg/kg/month are given.

Preparations of IgG for replacement contain predominantly IgG1 and IgG2; the amounts of IgG4 in most preparations are small, and in some IgG3 is absent. Nevertheless patients with selective IgG subclass deficiency, with or without IgA deficiency, may benefit from IgG replacement which provides high titres of antibodies that such a patient is unable to make. Neither the precise indications nor the dosage for such therapy has been well established.

Immunoglobulin replacement therapy by subcutaneous infusions of gammaglobulin is used increasingly. The results indicate that this type of treatment is well tolerated with a very low frequency of systemic adverse reactions.

Untoward reactions to infusions of immunoglobulin may occur. These include dyspnoea, flank pain, hypotension, collapse, fever, rashes or rigours; severe wheeze, chest pain or symptoms of anaphylaxis are indications for the infusion to be stopped and treatment to be given immediately (antihistamines and hydrocortisone i.v. or adrenaline s.c.). Most reactions are probably due to immunoglobulin aggregates, with only a very few being attributable to antibodies to IgA. Reactions tend to occur more frequently in severely hypogammaglobulinaemic patients, particularly at the initiation of treatment, and in those with intercurrent infections. Many reactions can be traced to excessively fast rates of infusion.

9.7.3 Enzyme replacement. Partial replacement of enzymes with frozen irradiated red blood cells has been attempted in infants with ADA or PNP deficiency. Apparently, the amounts of purine degradation enzymes within the red cells are not sufficient to permit efficient degradation of toxic metabolites within lymphocytes. Partial enzyme replacement in ADA deficiency has also been attempted by the use of bovine ADA modified by conjugation with polyethylene glycol. Repeated weekly administration of the conjugated enzyme resulted in marked clinical and immunological improvement in several patients.

Since the gene for ADA has been cloned, it has become possible to express it in T cells with a retroviral vector. This has provided the basis for the attempt at gene therapy in patients with ADA deficiency. This therapeutic approach is at present still under study. Transplantation of HLA-identical bone marrow remains the treatment of choice.

9.7.4 Blood transfusions. Blood transfusions should never be given to patients with cell-mediated immunodeficiency, unless fully oxygen saturated blood has been irradiated to eliminate viable white blood cells that may inappropriately engraft the patient. Blood transfusion is also safe when processed by freezing and centrifugation. However, lymphocytes are still viable in outdated blood, washed red blood cells, unprocessed plasma, and platelet preparations.

9.7.5 Treatment of opportunistic infections. Individual infections should be treated early with full doses of antimicrobial agents. Where possible narrow spectrum drugs selected on the basis of microbial sensitivity testing should be used. While prophylactic antibiotics may be useful, they increase the hazard of infection with fungi or other resistant organisms. Long-term treatment with combination sulfa drugs (co-trimoxazole, sulfamethoxazole-trimethoprim) and itrakonazole is believed to be of some benefit, but this has not been critically evaluated.

Antiviral agents such as acyclovir and gancyclovir have proven valuable in the treatment of some patients with persistent or severe viral infections.

9.7.6 Gastrointestinal disorders. Intestinal disease is frequent in ID patients and, in addition to treatment of infection or infestation, disaccharide or gluten-free diets may be of benefit in patients with sprue-like symptoms. In some instances intravenous hyperalimentation may be justified. *Giardia lamblia* and Campylobacter are frequent causes of diarrhoea, steatorrhoea or weight loss in ID. Treatment with atabrine or metronidazole is effective for giardiasis.

10 IMMUNODEFICIENCY ASSOCIATED WITH OR SECONDARY TO OTHER DISEASES

Table 9 lists some of the many congenital and hereditary conditions in which immunodeficiency has been described.

10.1 Chromosomal instability and defective repair

The immune system is dependent upon rapid and accurate lymphocyte differentiation and replication. Any syndrome associated with chromosomal instability or defective repair, such as ataxia-telangiectasia (9.4.2) can be expected to have associated immunological defects. The following syndromes fall into this category.

10.1.1 Bloom syndrome. Low birth weight, retarded growth, rashes from light sensitivity, well demarcated hyper- and hypo-pigmented skin lesions, molar hypoplasia and facial telangiectasia, characterize this rare autosomal recessive chromosomal instability syndrome that has been mapped to 11q23. Immunodeficiency with frequent infections, increased susceptibility to malignancies, reduced T-cell function and decreased serum IgM are found. At times serum IgG and IgA may also be diminished. IgM+ B cells are normal in number; the defect appears to be in B-cell maturation to IgM secretion. NK cell defects have been described.

10.1.2 DNA ligase I defect. A patient with growth retardation, sun sensitivity, immunodeficiency and defective DNA repair, phenotypically similar to Bloom syndrome (10.1.1) was found to have mutations in the DNA ligase I gene. The defect was not found in cells from patients with Bloom syndrome. The DNA ligase I defect thus appears to be a distinct entity.

10.1.3 Xeroderma pigmentosum. Is another photosensitive dermatosis. From infancy patients with this rare autosomal recessive condition have a marked sensitivity to sunlight and develop striking skin lesions – erythema, bullae, telangiectasia, keratoses, basal and squamous cell carcinomas. They are unable to repair UV damage to their DNA. A small number (>5%) of the affected children have recurrent infections and a demonstrable immunodeficiency with a decrease in CD4+ cells. Their sera reportedly contain antibodies which suppress T-cell (and possibly NK-cell) function. Serum IgG levels may be diminished. Cockayne's Syndrome and Trichothiodystrophy, similar photosensitive dermatoses, have not yet been described as having any immune defect.

10.1.4 Fanconi anaemia. This autosomal recessive syndrome is characterized by multiple organ defects including bone marrow failure, hyper-pigmentation and cafe au lait spots, limb defects (radial hypoplasia), genitourinary anomalies, abnormal facies (microphthalmia, micrognathia, broad nasal base, epicanthal folds) and chromosomal breaks. There is an increased incidence of leukaemia. Decreased T-lymphocyte and NK-cell function and decreased serum IgA concentrations have been described.

10.1.5 ICF syndrome. ICF is an acronym for the *i*mmunodeficiency, centromeric instability, *f*acial anomalies that characterize this syndrome. It is readily recognized by characteristic ocular hypertelorism, flattened nasal bridge, epicanthic folds, tongue protrusion and micrognathia. The immunodeficiency presents with recurrent sinopulmonary, gastrointestinal and skin infections. Generally, but not uniformly, serum IgM, IgG and IgA are decreased. Combined immunodeficiency has been described. Immunoglobulin administration may reduce infections in some patients. Variable degrees of mental retardation occur. The diagnostic finding is abnormal condensation of heterochromatin in chromosomes 1, 9, and 16 with increased frequency of mitotic recombination and the formation of multibranched chromosomes. This is associated with localized hypomethylation of classical satellite DNA. Inheritance is probably AR.

10.1.6 Nijmegen breakage syndrome. This condition presents with microcephaly at birth, short stature, a 'bird-like' facies and recurrent respiratory tract infections. About half the reported patients are mentally retarded. Immunoglobulin levels and T-cell function are decreased. The immune defect appears to be more severe than in Ataxia-Telangiectasia. Multiple structural chromosomal rearrangements are found preferentially in chromosomes 7 and 14. There is increased sensitivity to ionizing radiation and X-rays. Seemanova syndrome is identical.

10.1.7 Seckel ('bird-headed' dwarfism) syndrome. This is another one of the many 'bird-like' facies syndromes reported to be associated with immunodeficiency. Seckel syndrome is characterized by dwarfism (intra-uterine in onset), a 'bird-head' facies, severe brain dysplasia, mental retardation and many skeletal anomalies. Increased chromosomal breakage has been described. Some of the affected individuals develop a hypoplastic anaemia, pancytopenia and decreased serum immunoglobulins.

10.2 Chromosomal defects

Of the many syndromes associated with stable chromosomal abnormalities, several are accompanied by immunodeficiency.

Table 9. ID associated with or secondary to other congenital or hereditary conditions

Chromosomal instability or defective repair
- Bloom syndrome
- Xeroderma pigmentosum
- Fanconi anaemia
- ICF syndrome
- Nijmegen breakage syndrome (also known as Seemanova syndrome)
- Seckel ('bird-headed' dwarfism) syndrome

Chromosomal defects
- Down syndrome (Trisomy 21)
- Turner syndrome
- Deletions or rings of chromosome 18 (18p- and 18q-)

Skeletal abnormalities
- Short-limbed skeletal dysplasia (short-limbed dwarfism)
- Cartilage-hair hypoplasia (metaphyseal chondrodysplasia)

Immunodeficiency with generalized growth retardation
- Schimke immuno-osseous dysplasia
- Dubowitz syndrome
- Kyphomelic dysplasia with SCID
- Mulibrey's nannism
- Growth retardation, facial anomalies and immunodeficiency
- Progeria (Hutchinson–Gilford syndrome)

Immunodeficiency with dermatological defects
- Ectrodactyly-ectodermal dysplasia-clefting syndrome
- Immunodeficiency with absent thumbs, anosomia and ichthyosis
- Partial albinism
- Dyskeratosis congenita
- Netherton syndrome
- Anhidrotic ectodermal dysplasia
- Papillon–Lefèvre syndrome
- Congenital ichthyosis

Hereditary metabolic defects
- Acrodermatitis enteropathica
- Transcobalamin 2 deficiency
- Type I hereditary orotic aciduria
- Intractable diarrhoea, abnormal facies, trichorrhexis and immunodeficiency (?Stankler syndrome)
- Methylmalonic acidaemia
- Biotin dependent carboxylase deficiency
- Mannosidosis
- Glycogen storage disease, Type 1b
- Chediak–Higashi syndrome

Hypercatabolism of immunoglobulin
- Familial hypercatabolism
- Intestinal lymphangiectasia

Other
- Hyper IgE syndrome (Job syndrome)
- Chronic muco-cutaneous candidiasis
- Hereditary or congenital hyposplenia or asplenia
- Ivermark syndrome
- Familial intestinal polyatresia

10.2.1 Down syndrome. Trisomy 21 (Down syndrome) is a relatively common condition characterized classically by dysmorphic facies with slanting palpebral fissures, a flattened occiput, flat nasal bridge, hypotonia, mental retardation (which may be very mild) and recurrent infections. There is a progressive decrease in serum IgM. The thymus may be dysplastic. An increase in CD8 T cells with an NK phenotype has been described; the NK cell activity however was low. Abnormal delayed hypersensitivity (CMI), antibody formation and cytokine production have been reported. Chromosome 21 carries the gene encoding the interferon receptor; trisomy 21 lymphocytes are more sensitive to interferon than normals. Since patients with Down Syndrome often have evidence of early ageing and because the immune dysfunction may be progressive, it has been postulated that the immune defect may represent 'early immunological senescence'. The phenotypic findings of Down syndrome are perhaps best explained by a contiguous gene defect.

10.2.2 Turner syndrome. These patients, who have generally an XO karyotype, present clinically with short stature, ovarian dysgenesis, transient lymphoedema, a webbed neck and broad chest. They often have recurrent infections, autoimmune diseases and increased numbers of malignancies. About half have immunodeficiency, with decreased serum IgG and IgM. T- and B-cell numbers and responses are usually within normal limits. Patients with variants of the Turner syndrome, including mosaics, may show the same features.

10.2.3 Deletions or rings of chromosome 18 (18p- and 18q-). Individuals with rings and/or deletions of the short or long arms of chromosome 18 may present with mid-facial hypoplasia or ptosis, mental retardation, growth deficiency. Half have been found to have markedly decreased serum IgA and some have IgG subclass deficiency and defective antibody formation.

10.3 Skeletal abnormalities

The known inter-relationship between new bone formation, lymphocytes and cytokines leads to the expectation of some forms of skeletal dysplasia in patients with immunodeficiency. Short-limbed skeletal dysplasia (dwarfism) has, for example, been described in patients with ADA deficiency (see 9.2.3). In the syndromes listed below skeletal abnormalities are striking features; immunodeficiency is frequently although not universally present. All are also associated with growth-retardation such as that described in Section 10.4.

10.3.1 Short-limbed skeletal dysplasia (short-limbed dwarfism). The preferred nomenclature is short-limbed skeletal dysplasia (SLSD). The term is used to describe a group of patients in whom stature is disproportionately reduced, with greater involvement of the limbs than the trunk. It has been reported in patients with ADA deficiency (see 9.2.3) and in SCID with normal ADA (see Section 9.2).

10.3.2 Cartilage-hair hypoplasia (metaphyseal chondrodysplasia). These patients present with short-limbed skeletal dysplasia and usually, though not always fine, sparse (hypoplastic) unpigmented hair and severe immunodeficiency. The inheritance is AR. In Finland, the incidence is approximately 1: 23,000 births. Multiple organ systems may be involved: ligamentous laxity, macrocytic anaemia, neutropenia, megacolon (including Hirschsprung's syndrome) have all been described. Most patients have frequent infections and demonstrably defective cellular immunity. The defects in cellular immunity may relate to abnormal intracellular signalling pathways or in a trans-acting factor which regulates the expression of several early activation genes in T cells. B-cell numbers and functions are normal. The gene has been mapped to 9p13. Bone marrow transplantation has fully corrected the immune deficiency, but does not influence the chondrodystrophy.

10.4 Immunodeficiency with generalized growth retardation

Generalized growth retardation is common in children with recurrent infections, malnutrition and chronic pulmonary disease. It is prominent in syndromes involving the endocrine or the gastro-intestinal tract. As noted in Sections 10.1 and 10.2, dwarfism is a common component of syndromes involving chromosomal abnormalities and those with skeletal dysplasia (10.3). The following syndromes are characterized by growth retardation as a presenting condition.

10.4.1 Schimke immuno-osseous dysplasia. Several patients have been described with skeletal dysplasia, pigment abnormalities (lentigenes) and nephropathy. The inheritance is AR. Most patients have recurrent infections with striking lymphopenia, especially of T (CD4+) cells. Mitogen responses and DTH are diminished. B-cell numbers and function are normal. The nephropathy is associated with circulating immune complexes.

10.4.2 Dubowitz syndrome. This rare AR condition is associated with pre- and post-natal dwarfism, distinctive facial dysmorphism and eczema. Bone marrow failure, with pancytopenia has been reported.

10.4.3 Hoyeraal–Hreidarsson syndrome. Two brothers and three additional unrelated boys with pre-natal growth retardation, cerebellar hypoplasia, microcephaly, developmental delay and progressive pancytopenia with combined immunodeficiency have been reported.

10.4.4 Kyphomelic dysplasia with severe combined immunodeficiency. This rare skeletal dysplasia with short angulated femora, bowed long bones, short ribs and metaphyseal abnormalities has been described in an infant with SCID.

10.4.5 Ischiadic hypoplasia, renal dysfunction and immunodeficiency. A child of consanguineous parents with pre-natal growth retardation, microcephaly, abnormal facies (flat-face, hypertelorism, epicanthic folds, strabismus, short nose, low set ears), syn- and polydactyly, ischiadic hypoplasia with hypospadias and cryptorchidism, renal dysfunction and hypogammaglobulinemia, which appears distinct from Dubowitz syndrome (10.4.2), has been described.

10.4.6 Mulibrey's nannism. Mulibrey is an acronym for *m*uscle, *l*iver, *b*rain and *e*ye, organs described to characteristically be involved in this AR syndrome. Affected patients have pre- and post-natal dwarfism, a triangular facies (with a J-shaped sella turcica) and characteristic retinal pigment changes. Many have associated dermal lesions (naevi flammel, angiomata). Early death, commonly from constrictive pericarditis (which may be relieved by surgical intervention) occurs. Growth hormone and antibody deficiency has been described in this form of dwarfism. Serum IgM and IgG were decreased, antibody responses were ablated, B-cell numbers decreased. Growth hormone administration resulted in increased growth but did not improve immunological responses.

10.4.7 Growth retardation, facial anomalies and immunodeficiency. A variety of other small case reports suggest that the combination of facial anomalies and growth retardation may be associated with recurrent infection. In some instances there are decreased immunoglobulins; in some neutropenia. The reports are insufficient at this time to categorize the clusters more clearly, but the finding of facial anomalies with growth retardation warrants immunologic investigation.

10.4.8 Progeria (Hutchinson-Gilford syndrome). Alopecia, short stature and loss of subcutaneous fat are the hallmarks of this rare syndrome. Skin fibroblasts have reduced ability to replicate. A described reduction in T (CD4+) cells and reduced IgG levels may relate to the rapidly accelerated aging process. A somewhat similar and very rare condition, the SmithMulvihill syndrome (also known as Shepard–Elliot–Smith–Mulvihill) presents with short stature, progeria, microcephaly with ocular and dental anomalies, and pigmented naevi. In some instances recurrent infections and diminished IgG levels and in one patient lymphopenia with diminished T and B cells were found.

10.5 Immunodeficiency with ectodermal dysplasia and other dermatological defects

As noted in previous sections, immunodeficiency is often associated with a variety of dermatological conditions, including some described as ectodermal dysplasia, a term that encompasses many conditions that are often not clearly differentiated. The following syndromes present primarily as dermatological problems.

10.5.1 Ectrodactyly-ectodermal dysplasia-clefting syndrome (EEC). These unusual and rare patients are recognized most often because of their clefting problems; lobster claw deformities of the extremities and cleft palate, that are present in the majority, but not all of the affected patients. The uniform finding is of ectodermal dysplasia involving the hair, skin, nails and teeth. Lacrimal duct atresia is common. They have recurrent respiratory, lachrymal and urinary tract infections. In one case a T-cell abnormality, which later resolved, was reported. A thymic abnormality resultant from the ectodermal defect was postulated. In other cases the immune system has been found normal and the recurrent infections thought to be secondary to the ectodermal defects per se. The ectrodactyly locus is at 7q21.3.

10.5.2 Immunodeficiency with absent thumbs, anosmia and ichthyosis. Several syndromes, for example the Fanconi syndrome (10.1.2), are characterized by radial dysplasia and/or absent thumbs. Three sibships have been reported with short stature, absent thumbs, anosmia, ichthyosis (with chronic mucocutaneous candidiasis) and recurrent, predominantly viral and fungal as well as bacterial infections. Serum IgA was absent; IgG and IgM was variably decreased. Mitogen responses were diminished.

10.5.3 Partial albinism and immunodeficiency. This rare autosomal recessive disease is characterized by partial albinism due to abnormal migration of melanosomes from melanocytes to keratinocytes. It is distinguished from Chediak–Higashi syndrome by the absence of giant granules. Patients have a propensity for fungal, viral and bacterial infections. Immunoglobulins and DTH may be decreased. Abnormal T-cell cytotoxicity and diminished NK-cell activity have been described. These patients have in addition to increased susceptibility to infection, a lymphoproliferative reaction similar to that seen in Chediak–Higashi syndrome that leads to early death. The defect has been corrected by bone marrow transplantation.

10.5.4 Dyskeratosis congenita. This disease is characterized by cutaneous pigmentation, nail dystrophy and oral leukoplakia. Inheritance can be X-linked, AR or AD. There is an increased risk of malignancy. Bone marrow failure frequently occurs in childhood with resultant increased infections, but variable immunological defects. Hypogammaglobulinaemia, is found in many patients, along with diminished cell-mediated immunity.

10.5.5 Netherton syndrome. A large group of patients presenting with a classic triad of trichorrhexis (invaginata and nodosa), congenital ichtyosis (linear circumflex, erythrodermia) and atopy have been described. The ichthyosis, which is present at birth, can be associated with profound hypernatraemic dehydration. Decreased *in vitro* lymphocyte responses to mitogens and negative skin tests to a battery of bacterial antigens may be found. Some have had abnormally low or high serum immunoglobulin levels.

10.5.6 Anhidrotic ectodermal dysplasia. This syndrome is characterized by hypohidrosis, faulty dentition and hypotrichosis. Most cases are X-linked recessive; a few are AR. Heterozygotic females may have partial symptomatology. Recurrent upper respiratory infection are a frequent problem. Although immunoglobulin levels and DTH have been described as abnormal in some patients, no consistent T- or B-cell abnormality has been found. Diminished chemotactic activity has been reported in a possibly related condition, congenital ichtyosis (see also 10.5.8).

10.5.7 Papillon–Lefevre syndrome. Hyperkeratosis of the hands and feet with periodontal disease leading to premature loss of teeth is in some cases associated with pyoderma. Neutrophil chemotaxis is often diminished. This syndrome needs to be distinguished from the leukocyte adhesion defects (12.2.1) and the Hyper-IgE (Job's) syndrome (10.8.1).

10.5.8 Congenital ichthyosis. The X-linked form of this disease is caused by a deletion in the steroid sulfatase gene on the short arm of the X chromosome, close to the gene associated with chronic granulomatous disease (see Section 12.3.1) at Xp21. Combined deletions of both occur.

10.6 Hereditary metabolic defects

Several hereditary metabolic defects other than adenosine deaminase and purine nucleoside phosphorylase deficiency can impair immune function. In the instances listed below the impairment of immune function may be only a minor component of the manifestations of the disease.

10.6.1 Acrodermatitis enteropathica. This autosomal recessive disease characterized by eczema, diarrhoea, and malabsorption has been reported in association with recurrent sinopulmonary infections, decreased serum Ig, intermittently reduced numbers and function of T cells and abnormal cell-mediated immunity. In some patients abnormal chemotaxis was found. The syndrome is attributable to zinc deficiency from defective gastrointestinal zinc absorption. Symptomatology responds dramatically to the administration of increased amounts of zinc given by mouth.

10.6.2 Transcobalamin 2 deficiency. Autosomal recessive defects in the vitamin B_{12} transport protein, transcobalamin 2, have been described. These defects impair the normally rapid cell proliferation required for haematopoiesis, lymphocyte proliferation and gastrointestinal tract epithelial cell regeneration. Affected infants present with diarrhoea, failure to thrive, megaloblastic anaemia, defective granulocyte function and immunodeficiency involving primarily B lymphocyte function. Administration of vitamin B_{12} in pharmacological doses rapidly reverses the signs and symptoms. Folinic acid may also be required.

10.6.3 Type I hereditary orotic aciduria. An autosomal recessive disease which presents with retarded growth, recurrent diarrhoea, megaloblastic anaemia, increased numbers of infections (including fatal meningitis and varicella), and lymphopenia with decreased numbers of T lymphocytes and impaired cell-mediated immunity.

10.6.4 Intractable diarrhoea, abnormal facies, trichorrhexis and immunodeficiency. Several patients have been reported with prenatal growth retardation, facial dysmorphism with hypertelorism, woolly, friable hair (trichorrhexis) and severe secretory diarrhoea. While serum immunoglobulin was normal, antibody responses were defective. *In vitro* lymphocyte responses to mitogens were likewise normal, but skin tests for delayed hypersensitivity (DTH) were diminished. Stankler Syndrome may be similar.

10.6.5 Methylmalonic acidaemia. Methylmalonic acidaemia is similar to Transcobalamin II deficiency; it represents a series of several distinct enzymatic defects that affect cobalamin (B_{12}) metabolism and result in the accumulation of excess levels of methylmalonic acid which inhibits bone marrow stem cell growth. Pancytopenia is common; B-cell numbers and serum IgG may be reduced. There may be no response to Vitamin B_{12}. Folic acid treatment may reverse the problem.

10.6.6 Biotin dependent carboxylase deficiency. Infants affected with this autosomal recessive condition present with convulsions, ataxia, alopecia, Candida dermatitis, keratoconjunctivitis and increased urinary excretion of beta-hydroxyproprionic acid. Isolated IgA deficiency and reduced numbers of peripheral T and or B lymphocytes have been reported. Biotin administration results in biochemical and clinical improvement.

10.6.7 Mannosidosis. This lysosomal storage disease resembles Hurler syndrome with abnormal facies, dysostosis, heparosplenomegaly and recurrent infections. The accumulation of the mannose-rich lysozomes may interfere with both neutrophil and lymphocyte function.

10.6.8 Glycogen storage disease, Type Ib. Patients with this variant of glycogen storage disease may have neutropenia and neutrophil dysfunction, presumably due to defective glucose metabolism. They have recurrent infections.

10.7 Hypercatabolism or loss of immunoglobulin
Many diseases are associated with hypercatabolism of Ig. These can be distinguished from failure of Ig production by metabolic studies. The following are some of the conditions in which hypercatabolism of Ig may lead to immunodeficiency.

10.7.1 Familial hypercatabolism. A kindred has been described with recurrent infections, bone abnormalities, abnormal glucose metabolism and diminished levels of serum albumin and immunoglobulin which could not be explained by increased gastrointestinal or urinary losses.

10.7.2 Intestinal lymphangiectasis. Losses of lymphocytes and immunoglobulins into the gut can result in significant lymphopenia, diminished cell-mediated immunity and decreased serum Ig levels.

10.8 Other

10.8.1 Hyper-IgE syndrome (Job syndrome). Recurrent (usually staphylococcal) abscesses that are often 'cold', lung abscesses which result in pneumatoceles, skeletal anomalies, coarse facies, eosinophilia and very high serum levels of IgE are characteristic of the Job or hyper-IgE syndrome. The B lymphocytes from these patients spontaneously produce large amounts of IgE *in vitro*. Several kindreds involving both males and females, and affected mothers or fathers with affected children have been reported, suggesting that in some instances the disease is autosomal dominant. Sporadic cases also occur. The immunological defect is not yet fully understood.

10.8.2 Chronic muco-cutaneous candidiasis. These patients have severe persistent candidal infections of skin and mucosa. They have markedly impaired cell mediated immunity to Candida antigens. The relationship, if any, between the specifically diminished T-lymphocyte responses and infection is not understood. A mannose deficiency has been noted in the monocytes of some patients. This condition is frequently associated with familial polyendocrinopathy. Severe secondary bacterial infections may supervene.

10.8.3 Hereditary or congenital hyposplenia or asplenia. Persons with hyposplenia or asplenia (whether post-traumatic, surgical, congenital or hereditary) are at increased risk for sepsis. Infants with congenital or hereditary asplenia are particularly prone to such infections. The responsible organisms are usually pyogenic, pneumococci being the most common. Increased susceptibility to intracellular parasites (for example, malaria) and some viral agents has also been reported.

10.8.4 Ivemark syndrome. This syndrome probably represents disturbed laterality including partial *situs inversus* during very early embryogenesis resulting in major defects in organ formation. In addition to cardiac defects, asplenia is found. The problem of infection are as described in Section 10.8.3.

10.8.5 Familial intestinal polyatresia. Multiple areas of atresia throughout the gastrointestinal tract characterize this AR syndrome. Several patients have had an associated severe combined immunodeficiency, all with markedly decreased immunoglobulin and variably decreased T-cell number and function, and reduced immunoglobulins. Patients with familial intestinal polyatresia require surgery; as these patients may have an underlying SCID, all blood given must be irradiated to prevent engraftment.

10.8.6 Immunodeficiency with thymoma. About 10% of patients with thymoma have an immunodeficiency, an association described in 1954. The disorder is seen most commonly in older (>40 years) adults and is generally identified by the radiological finding of the thymoma and on subsequent study by hypogammaglobulinaemia. The immunodeficiency often initially appears similar to common variable immunodeficiency (9.3.7). The immunodeficiency may precede the thymoma. The types of infections (candidiasis, herpes), the frequent occurrence of autoimmune disease (myasthenia gravis, pernicious anaemia, diabetes, thrombocytopenia) and studies of cellular responses indicate that T cells as well as B cells are affected; the condition thus fits the criteria for a combined immunodeficiency (see Section 9.2). The occurrence is sporadic and affects both sexes. No familial cases have been reported. Immunoglobulin administration decreases the number of infections. Surgical removal of the thymoma may meliorate some symptoms, but does not cure the immunodeficiency. If the thymoma is malignant, death usually follows within a few years of diagnosis.

11 COMPLEMENT DEFICIENCY

11.1 Complement system
The classic complement system consists of nine numbered components (Cl to C9) and five regulatory proteins (Cl inhibitor, C4 binding protein, properdin, Factors H and I). The first component (Cl) comprises three subcomponents, Clq, Clr, and Cls. It is the molecular interaction between Clq and aggregated IgG or IgM (as in an antigen-antibody complex) that initiates activation of the classic complement sequence. The fixation of Clq activates Clr and Cls. Cls cleaves C4 and C2, whose active fragments C4b and C2a form the classical pathway C3 convertase.

An alternative pathway to C3 activation consists of C3b, Factor B and Factor D. Factor B binds to a cleavage fragment of C3, C3b, to form C3bB. Factor D cleaves the bound Factor B to form the alternative pathway C3 convertase (C3bBb). It activates C3 in a fashion similar to the C3 convertase of the classical pathway, C4b2a. Properdin appears to stabilize this alternative pathway C3 convertase.

A large number of biological activities important in the inflammatory response and in host resistance to infection take place at various steps in complement activation. The lytic property for bacterial or animal cells, however, requires the activation of C5 to C9 by the classical or alternative pathway. The enhancement of phagocytosis by complement is probably of great biological significance and requires the deposition of C3b or iC3b on the particle to be ingested. Certain viruses are neutralized after interaction with antibody and only the first two complement components (Cl and C4); other viruses require C2 and C3 in addition. Immune adherence, a property whereby antigen–antibody complexes adhere to complement receptor 1 (CR1), occurs with complement activation through the C4 and C3 steps. The ligands for CR1 are C4b and C3b. Histamine release from mast cells, smooth muscle contraction and increased vascular permeability caused by anaphylatoxin activity are properties of each of the two small fragments (C3a and C5a), which are released when C3 or C5 are cleaved by their respective convertases. These fragments are also chemotactic for polymorphonuclear leukocytes, particularly C5a, which also causes exocytosis of neutrophils. The classic complement pathway appears to be important in the dissolution of immune complexes.

Complement enhances B-cell activation at two distinct stages of development within the lymphoid compartment: (a) formation of the PALS (periarteriolar lymphoid sheath)-associated foci; and (b) survival within the germinal centre (GC). The former involves reduction in the threshold of B-cell signalling via the CD21/Tapa-1 complex; the latter involves increased adhesion between the B cells and the follicular dendritic cells, mediated by CD21 and C3d-antigen in germinal centre interactions.

11.2 Genetic defects in human complement

Genetic defects have been described for almost all the complement components in humans, including Clq, Clr (and Cls), C4, C2, C3, C5, C6, C7, C8 and C9 deficiency (Table 6). In all these instances defects are transmitted as phenotypically autosomal-recessive traits, and the heterozygotes can usually be detected because their sera contain approximately half the normal level of the deficient component as determined by functional and/or immunochemical tests. Non-functional variants of Clq have been described. C8 deficiency is unusual in that the β chain is not covalently associated with the α and γ chains. Thus affected Caucasian C8 deficients lack the β chain and black C8 deficients lack the α, γ chains. Both forms have non-functional, incomplete C8 molecules in their serum. C9 deficiency has a very high incidence in Japanese. Genetic deficiencies in the alternative pathway are very rare. Deficiency of properdin is X-linked. The mode of inheritance of Factor D deficiency is not entirely clear.

Genetic defects have also been recognized for three inhibitors of the complement system: C1 inhibitor, Factor I and Factor H. Deficiency of the C1 inhibitor is inherited as an autosomal dominant. This deficiency is associated with hereditary angioedema (HAE), or Quincke's disease. In 15% of affected kindred the sera contain normal or elevated amounts of an immunologically cross-reacting (CRM+), non-functional protein due to missense point mutations in the C1 inhibitor gene in the exon encoding the active site. In the majority of affected kindred the defects are due to nonsense mutations or unequal crossovers in the Alu sequences of introns 4, 5, 6, 7 and 8.

The genes for factor B, C2 and C4 are located on the short arm of chromosome 6 between HLA-D and HLA-B. The C4 gene is duplicated and the two genes are designated *C4A* and *C4B*; C4A molecules usually bear the Rodgers blood group substance and C4B the Chido blood group substance. Complete C4 deficiency is very rare and occurs only when all four alleles (the two of C4A and the two of C4B) are not expressed. In one case, this was due to isodisomy of a paternal chromosome 6p that was deficient in *C4A* and *C4B* (C4AQO and C4BQO). Thirty-five per cent of individuals in all racial groups lack one to three C4 alleles. Those with C4AQO have a high incidence of SLE and juvenile rheumatoid arthritis. The genes for factor B and C2 are so tightly linked that no crossover has yet been observed between them, but unequal crossover in the MHC may result in the expression of three C4A alleles and one C4B allele, or vice versa.

Genetic polymorphisms are known for C4A, C4B, C2, Factor B, C3, C6, C8α, and C8β. Polymorphic variants of C5, C7, Factor D, Factor H, Factor I and C1 inhibitor are rare.

All patients with complement deficiency are more or less unduly susceptible to infection and to development of immune complex disease. For example, patients with C1 inhibitor deficiency (HAE) have prominent angioedema but are also prone to develop immune complex disease.

Impeded androgens have proved extremely effective in the treatment of hereditary angioedema. Purified C1 inhibitor preparations are available for intravenous administration and should be used in the treatment of acute attacks of angioedema. There is no satisfactory replacement therapy for the other complement deficiencies, largely because the catabolic rate of these proteins is very high. Sometimes patients with late component deficiencies require anti-microbial prophylaxis or immunizations because of recurrent neisserial infections.

12 DEFECTS OF PHAGOCYTE NUMBER AND FUNCTION

Apart from congenital neutropenia, which has several causes, there may be inherited defects of phagocyte function, affecting polymorphonuclear and/or mononuclear phagocytes. Neutrophil function depends on movement in response to chemotactic stimuli, adherence, endocytosis, and killing or destruction of the ingested particles. Mobility depends on the integrity of the cytoskeleton and the contractile system; directional mobility is receptor mediated. Endocytosis depends on the expression of certain membrane receptors, for example, for IgG, C3b and iC3b, and on the fluidity of the membrane. Congenital defects of phagocyte number and/or function and their associated features are listed in Table 5.

Assays for chemotaxis can be performed by the use of Boyden chambers or migration under agarose; defects of contractibility, however, can only be assessed with Boyden chambers. The measurement of nitroblue tetrazolium (NBT) dye reduction by actively phagocytosing leukocytes has been accepted as a standard measure for the adequacy of super oxide production. Better suited for detection of partial defects are more sensitive assays including chemiluminescence and the direct measurement of superoxide. Assays for bacterial killing are demanding and yield highly variable results depending on the bacterial species used in the assay.

12.1 Congenital neutropenias

12.1.1 Severe congenital neutropenia (SCN, Kostmann syndrome). SCN is characterized by profound neutropenia (<200/μl) and a maturation arrest of myeloid progenitor cells at the promyelocyte–myelocyte stage. Pharmacological doses of G-CSF (ranging from 0.8 to 70 μg/kg/day) increase the neutrophil count in the majority of patients and decrease infections (mainly otitis, stomatitis and pneumonia) significantly.

While most SCN patients have normal G-CSF receptors, a subgroup presents with non-sense G-CSF receptor mutations truncating the C-terminal cytoplasmic region crucial for maturation signalling. Patients in this subgroup develop myelodysplastic syndrome and acute myeloid leukaemia. They should not be treated by G-CSF, but probably by bone marrow transplantation if a donor is available.

12.1.2 Cyclic neutropenia. Cyclic neutropenia is characterized by 21-day oscillations of blood counts with neutrophil levels fluctuating between the lower limit of normal and 0. During the periods of severe neutropenia patients are prone to severe infections, but otherwise are relatively well. G-CSF in doses of 1–5 μg/kg/day raises the oscillating neutrophil counts and shortens the periodicity of the cycles which persist. None of the patients developed MDS/AML.

12.2 Defects of motility

12.2.1 Leukocyte adhesion defects (LAD). A large number of cases has been described with a defect in the iC3b receptor of phagocytes (CD11b), the C3dg receptor of phagocytes called p150.95 (CD11c) and the LFA-1 (CD11a) adhesion molecule of T lymphocytes, NK cells and phagocytes. This deficiency results from abnormal biosynthesis of a 95 kd β chain (CD18), which is common to the iC3b receptor, p150/95 and LFA-1; the gene encoding the beta chain maps to chromosome 21. This defect has been called leukocyte adhesion defect type 1 (LAD1). It is inherited as an autosomal recessive disorder. The phenotypic expression of the leukocyte adhesion defect is variable. In the severe phenotype <1% of normal adhesion molecules are expressed whereas in the moderate phenotype up to 10% of these molecules are expressed. Patients have defects in mobility, adherence and endocytosis. They usually present with infections of skin, periodontitis and intestinal or perianal fistulas. In the severe phenotype omphalitis, delayed umbilical cord separation, septicaemia and massive leucocytosis (up to 100,000 mm^3) are typical.

A second type of leukocyte adhesion deficiency (LAD2) has been described in unrelated Palestinian children. These infants are unable to synthesize fucose from GDP mannose so that they cannot form the Sialyl-Lewis x ligand for the selectin molecules. The phenotype in this form of LAD is similar to the common form of LAD except that short stature, mental retardation as well as Bombay (hh) blood group has been noted in the former. It is inherited as an autosomal recessive. The enzyme defect has not been precisely defined and its chromosomal location is not yet known.

12.2.2 Chediak–Higashi syndrome (CHS). This autosomal recessive disease is characterized by partial oculo-cutaneous albinism due to dysmaturation of melanosomes. Giant granules are found in all nucleated cells. Besides neutropenia abnormalities of granulocyte and monocyte mobility and chemotaxis, as well as defective NK-cell cytotoxicity are demonstrable. The latter may explain an EBV-associated lymphoproliferation resembling familial lymphohistiocytosis (FLH) that leads to pancytopenia and hypofibrinogenemia following macrophage activation. The CHS gene has been identified and encodes a protein which may regulate microtubule-mediated lysosome transport.

12.2.3 Specific granule deficiency. Neutrophils have two types of granules which contain a variety of enzymes. In a few patients described to have abnormal neutrophil structure (bi-lobed nuclei), specific (secondary) granules (which normally contain lactoferrin and receptors for chemotactic factors as well as for iC3b and C3dg) are incompletely formed. Defective chemotaxis has been described. Clinically there are increased numbers of skin infections and progressive pulmonary disease. The precise nature of the defect is unknown.

12.2.4 Shwachman syndrome. Hereditary pancreatic insufficiency associated with neutropenia, defective neutrophil mobility and chemotaxis, thrombocytopenia and anaemia and often metaphyseal chondrodysplasia with short stature are the principal features of this syndrome. Affected infants have recurrent pyogenic sinopulmonary and skin infections and may have hypogammaglobulinaemia. It is inherited as an AR.

12.2.5 Other. Phagocyte function may also be defective in a number of generalized hereditary diseases, such as glycogen storage disease type Ib. The phagocytic dysfunction does not constitute a characteristic or diagnostic feature of these diseases. Certain immunodeficiency syndromes may be associated with a constant primary (e.g. the Wiskott–Aldrich syndrome) or a fluctuating secondary chemotactic defect.

12.2.6 Treatment. Infections in phagocyte deficiencies should be treated with appropriate antibiotics, surgery, and in case of septicaemia, neutrophil transfusion. In case of defects of intracellular killing lipophilic antibiotics able to penetrate phagocytes are preferable. Long-term infection prophylaxis by sulfamethoxazole-trimethoprim (antibacterial) and itraconazole (anti-aspergillus) is valuable, especially for CGD. If neutrophils are transfused, cells should be mobilized in the donors by G-CSF, and should be protected in the recipient from apoptosis by G-CSF as well. Patients with congenital neutropenias (e.g. SCN, cyclic neutropenia) and the neutropenia associated with the hyper-IgM syndrome profit from G-CSF treatment. G-CSF must not be given to a subroup of SCN having G-CSF receptor mutations predisposing to MDS/AML.

Some CGD patients (e.g. with mutations still allowing residual O_2^- production) may profit from interferon γ treatment. Bone marrow transplantation has been successful in patients with SCN, LAD, CHS and CGD.

12.3 Defects of microbial killing

12.3.1 Chronic granulomatous disease (CGD). Defects in intracellular killing of ingested micro-organisms usually result from failure of production of superoxide union, singlet oxygen, and hydrogen peroxide. This failure results in chronic granulomatous disease (CGD). The organisms cultured from lesions of patients with CGD are generally catalase-producing and include Staphylococci, *E. coli*, *Serratia marcescens*, Nocardia, fungi, such as Aspergillus, and other organisms with formation of chronic infected granulomas, especially of lymph nodes, liver, lung and gastrointestinal tract. The reaction $NADPH + 2O_2 \rightarrow NADP + 2O_2^- + H^+$ requires NADPH oxidase, a multi-component enzyme localized in the plasma membrane of phagocytic leukocytes. The enzymatic unit of NADPH oxidase consists of a flavo-heme protein called cytochrome b558, a heterodimer composed of a 91 kD chain (gp91phox) and a 22 kD chain (p22phox). When phagocytes are activated, a number of cytosolic oxidase components translocate to the membrane and induce enzymatic activity by a conformational change in the flavocytochrome. X-linked CGD results from a defect in gp91phox. In some cases, CGD is associated with a defined deletion in the short arm of the X chromosome at Xp21. In some cases of autosomal recessive CGD, p22phox whose gene is encoded on chromosome 16 is defective; in the others one of two cytosolic components of NADPH oxidase, p67phox or p47phox, is defective.

12.3.2 Neutrophil G6-PD deficiency. Glucose-6-Phosphate Dehydrogenase (G6-PD) is a necessary component of the hexose monophosphate shunt. The G6-PD gene, located at Xq28, is prone to frequent mutations; over 200 variants have been recorded. In Neutrophil G6-PD deficiency the variant leads to a severely defective enzyme and, because of its function in NADPH generation, results in reduced intracellular H_2O_2 production on leukocyte activation. As in CGD, there is failure in the killing of catalase positive intracellular organisms. The clinical presentation is the same as in CGD except that it occurs at a later age. Since NBT cannot be reduced, the NBT test can be used for ascertainment. Reduced G6-

PD in red blood cells causes concomitant chronic haemolytic anaemia.

12.3.3 Myeloperoxidase deficiency. Myeloperoxidase is one of the more abundant enzymes in polymorphonuclear leukocytes. The gene is located at 17q22-23. Deficiency is not uncommon (1:2000 to 4000 in the USA) and is usually clinically silent. Granulocytes lacking the enzyme fail to kill Candida: some affected persons (presumably having a more defective mutation and often in association with other diseases) have suffered from severe, recurrent candidal infections.

12.3.4 Interferon-γ receptor deficiency. Interferon-γ is the major cytokine responsible for the activation of macrophages. It primes macrophages for production of reactive oxygen intermediates and nitric oxide (NO). Absence of the α-chain of the interferon-γ receptor can be a cause of selective susceptibility to BCG, atypical myobacteria or salmonella infection. Giant cells and granulomas are not found. Susceptibility to viral infections seems normal.

FOOTNOTES

[a]These determinations require special facilities and can be arranged by writing to Dr H. D. Ochs, Department of Pediatrics RD-20, University of Washington, Seattle, WA 98195, USA.

[b]Obtainable from the Institute Pasteur-Merieux, Lyon, France.

[c]Antigen and assay obtainable from Dr M. Eibl, Institute of Immunology, Borschkegasse 8a, 1090 Vienna, Austria.

[d]Obtainable from Merck, Westpoint, PA 19486, USA.

[e]Obtainable from Hollister-Stier Labs, Box 3145, Terminal Annex, Spokane, WA, USA.

[f]Undiluted glycerin-free Dermatophytin (Trichophyton), Hollister-Stier Labs, Box 3145, Terminal Annex, Spokane, WA, USA.

[g]Mumps skin test, Eli Lilly & Co, Indianapolis, IN 46206, USA.

[h]Paediatric diphtheria and tetanus toxoid, Wyeth Laboratories, P.O. Box 8299, Philadelphia, PA 19101, USA.

Leukemic Cells from Progressive B-CLL Respond Strongly to Growth Stimulation *In Vitro*

Miguel Aguilar-Santelises[1], Håkan Mellstedt[2], and Mikael Jondal[1]

[1]Department of Immunology, Karolinska Institute, and [2]Department of Oncology, Radiumhemmet, Karolinska Hospital, Stockholm, Sweden

Isolated leukemic B cells from patients with B-chronic lymphocytic leukemia (B-CLL) were tested for proliferative response *in vitro* to *Staphylococcus Aureus* strain Cowan 1 (SAC), IL-2, and low molecular weight (MW) BCGF. Patients were classified according to clinical stage and progressiveness. Ten of eighteen cell populations from patients with progressive B-CLL responded *in vitro* with a stimulation index (SI) >20. Only 1/16 non-progressive patients had a proliferative but low response. Normal unfractionated tonsillar B cells responded to SAC and BCGF, whereas normal high buoyant density B cells were unresponsive. After 3 days of stimulation, responding B-CLL cells had multiplied and the B cells expressed CD5, CD19, and weakly CD21. No cells in the responding cultures exhibited CD3 or the EBV nuclear antigen EBNA-1. Cell maturation, measured as IgM secretion, was found in some, but not in all responding B-CLL cultures. Thus, B-CLL cells from patients with progressive disease have the capacity to respond to signaling through surface Ig receptors and to certain T-cell factors which was not the case for B-CLL cells from non-progressive patients. The pattern of *in vitro* response may be related to disease progression, reflecting a dependency of normal immunoregulatory mechanisms and/or a dysregulation of the growth control in the leukemic cells.

INTRODUCTION

B-chronic lymphocytic leukemia (B-CLL) has a variable clinical course, from a benign, indolent condition (1) to a rapidly fatal disorder (2,3). The extent of the disease is described by staging systems (4,5) and progressiveness by the development of anemia, thrombocytopenia, tissue infiltration, number of leukemic cells in peripheral blood, and constitutional symptoms (6). Clinical progression is related to growth of the leukemic cells and is reflected by an increased number of cycling cells in the blood (7–9). Whether the leukemic expansion occurs at the stem cell level in lymphoid tissues or in the entire B-CLL population, including cells in blood, is not clear (10,11).

B-CLL cells express CD5 belonging to a subpopulation of B cells which are elevated in autoimmune diseases (3). The frequency of autoantibodies and autoimmune disorders in B-CLL is high, and secreted IgM, derived from the leukemic cells, often reacts broadly with different potential autoantigens (12,13).

There is evidence that T and NK cells are activated in B-CLL (3,14–17) and predominant T-cell clones have been detected in benign cases of B-CLL by probing for rearranged beta chains of the T-cell receptor (TCR) (18). It has been suggested that B-CLL cells may be regulated by the normal T-cell compartment, both negatively by suppressor T cells, and positively by helper T cells (14,16). Tötterman *et al*, (14) have found evidence for chronic antigenic stimulation by accumulation of memory helper/inducer $CD4^+$ T cells, and we found a selective increase of soluble CD4 in serum of patients with progressive disease (19). Positive regulation of B-CLL cells by helper T cells may reflect normal mechanisms for B-T cell interaction (20), as B-CLL cells express MHC class II molecules (15,21,22) and may function as antigen-presenting cells (23).

If positive signaling through membrane IgM, or by helper T cells, are important events in B-CLL progression, the leukemic cells may be responsive *in vitro* to signaling through antigen-specific membrane IgM and to soluble factors. In a number of earlier studies, the response of B-CLL to factors such as BCGF, IL-1, IL-2, IL-4, IL-6, TNF, interferons and drugs, as well as anti-IgM antibodies, have been tested (3,9,10,15,24). From these studies it seems as if B-CLL cells are heterogeneous in their responsiveness, both with regard to differentiation and proliferation. However, the relationship between clinical activity and the *in vitro* response is not clear.

In the present study we compared the proliferative *in vitro* response of isolated B cells from blood of progressive or non-progressive B-CLL patients to *Staphylococcus Aureus* strain Cowan 1 (SAC), and the T cell factors IL-2, and low molecular weight (MW) BCGF. We found frequent and strong *in vitro* responses in B-CLL cells from patients with progressive disease, measuring cell numbers and DNA synthesis. The proliferative responses were, or were not, associated with cell differentiation as detected by IgM secretion.

MATERIAL AND METHODS

Patients

Thirty-four B-CLL patients, 15 women and 19 men, with a mean age of 73.4 ± 7.4 years (range 55–86 years) were studied (Table 1). The patients were staged according to Rai *et al*. (4). All patients had a blood lymphocyte count >4 × 10^9/l including a monoclonal B-cell fraction (κ or λ light chains). The diagnostic criteria have been described in detail elsewhere (25). Patients in stage 0 did not receive any treatment. No patient had received therapy within 30 days before test. Patients were considered to have a progressive disease, according to a modification of the criteria by the NCI Committee (26), if there was a progression during the preceding 3 months in disease-related anemia (Hb <100 g/l), thrombocytopenia (platelet count <100 × 10^9/l), and/or in spleen/liver/lymph node size (evaluated both by clinical examination and computer tomography of the abdomen), and/or more than a doubling of the blood lymphocyte counts, and/or appearance of constitutional symptoms. When these criteria were not fulfilled, B-CLL patients were defined as having non-progressive or stable disease.

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Correspondence to: Dr Miguel Aguilar-Santelises, Microbiology and Tumor Biology Center (MTC), Karolinska Institute, S-171 77, Stockholm, Sweden.

LEUKEMIA

Table 1 Clinical Characteristics of the B-CLL Patients

Patient Code	Sex	Age	Stage	Disease Progression	Months Since Diagnosis	Previous Therapy[a]	Blood Lymphocyte Count (X 10⁹/l)
L 45	F	78	0	–	237	–	40
L 63	F	83	0	–	180	–	62
L 65	M	78	0	–	72	–	45
L 71	F	75	0	–	74	–	50
L 84	M	69	0	–	66	–	55
L 86	M	76	0	–	75	–	50
L 140	F	85	0	–	72	–	32
L 167	F	60	0	–	60	–	36
L 172	M	77	0	–	210	–	34
L 174	F	72	0	–	60	–	11
L 82	M	68	I	–	48	–	70
L 150	F	66	I	–	12	–	62
L 159	F	68	I	–	108	–	16
L 164	M	81	I	–	6	–	38
L 170	M	78	I	–	228	+ (26)	28
L 173	M	57		–	156	+ (144)	90
L 50	F	62	I	+	3	–	127
L 68	F	81	I	+	18	+ (1)	15
L 73	F	76	I	+	96	+ (2)	31
L 160	F	73	I	+	98	–	129
L 169	F	72	I	+	66	–	120
L 62	M	66	II	+	42	+ (1)	160
L 133	M	72	II	+	78	+ (10)	170
L 141	M	73	II	+	30	+ (18)	30
L 153	M	86	II	+	3	–	209
L 156	M	77	II	+	38	–	240
L 87	F	77	III	+	120	+ (15)	40
L 161	M	79	III	+	120	+ (9)	85
L 162	F	78	III	+	49	+ (5)	500
L 19	M	66	IV	+	84	–	212
L 61	M	74	IV	+	72	+ (3)	10
L 69	M	75	IV	+	78	+ (8)	275
L 70	M	77	IV	+	120	+ (1)	73
L 158	M	72	IV	+	19	+ 18)	300

[a]Months since last treatment in brackets.

Reagents

Recombinant IL-2 was obtained from Du Pont Scandinavia (Stockholm, Sweden) and used at a concentration of 100 U/ml. SAC was bought from Calbiochem, San Diego, CA, USA and used at a final concentration of 0.005%. Low MW BCGF was purchased from Cellular Products, Buffalo, NY, USA and used at a 10% v/v final dilution. Propidium Iodide and RNAse were obtained from Sigma (St Louis, MO, USA). 1,2-phenylenediamine, dihydrochloride (OPD) tablets, as well as peroxidase-conjugated rabbit immunoglobulin anti-human IgM were from Dakopatts (Copenhagen, Denmark).

Cell Isolation and Immunofluorescent Staining

Heparinized venous blood was collected in vacutainer tubes (Becton Dickinson, Mountain View, CA, USA) and sedimented with 6% gelatin in isotonic salt solution for 45 min to obtain the leukocyte-rich plasma. The cells were incubated with carbonyl iron for 30 min at 37°C and phagocytic cells were removed by a magnet. Lymphocytes were obtained by Lymphoprep (Nycomed, Oslo, Norway) centrifugation. Cell samples were depleted of T lymphocytes by two consecutive cycles of roseting with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide (AET). Isolated cells were phenotyped by immunofluorescence staining using monoclonal antibodies anti-IgM, anti-MAC 387, phycoerythrin (PE)-conjugated anti-CD19, and PE-conjugated anti-CD25 from Dakopatts, PE-conjugated-anti-CD5 and FITC-IgG1/PE-IgG2a conjugated Simultest (Becton Dickinson) and FITC-F $(ab')_2$ fragments of affinity purified sheep antibodies anti-mouse IgG, not cross-reactive to human IgG (Cappel Research Reagents; Organon Teknika, Belgium). Non-stained cells and cells treated with PE-conjugate or FITC-conjugate alone were used as controls. Stained samples were analyzed in a Becton Dickinson FACScan system, using 10000 cells for each determination and an acquisition rate lower than 400 cells/s. Isolated non-roseting, leukemic B cells contained <0.2% $CD3^+$ cells (n = 18) and non-roseting normal unfractionated tonsillar B cells contained <0.2% $CD3^+$ cells (n = 3) (data not shown).

Culture Conditions

Isolated B cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 5% FCS. A final concentration of 2 × 10^6/ml was used in 96-microwell plates in the presence or absence of the stimulatory agents for

72 h at 37°C. Cells were then pulsed for 16 h with 1 μCi/well of ^{3}H-thymidine, harvested on glass fiber filters (Skatron, Sterling, VA, USA), dried and counted in a Beta counter (1218 Rackbeta; LKB Wallac) by addition of Optiphase, Hisafe II (LKB Scintillation Products, Loughborough, Leics, UK). Cells isolated from tonsils either as unfractionated B cells or as a subpopulation of 'high buoyant density' normal B cells (65% density by Percoll gradient) were used as control cell populations. ^{3}H-thymidine incorporation is expressed as stimulation index (SI) representing the ratio between c.p.m. obtained in stimulated culture and c.p.m. of unstimulated culture of the same cell population. All measurements were done in triplicate and only experiments with a mean error deviation <5% were accepted.

^{3}H-thymidine incorporation, cell numbers, viability, cell cycle position, and IgM secretion were measured simultaneously by culturing the leukemic cells in 12-well plates at 2 × 10^6/ml using 5 ml/well for 72 h. Two hundred microliter cell suspension was then transferred to 96-microwell plates and ^{3}H-thymidine incorporation measured in triplicates using a 16-h pulse. At 72 h, supernatants were harvested for IgM measurements, cells counted, and cell cycle positions analyzed. In long-term cultures (L 160 and L 158), cells were kept in 12-well plates and fed every fourth day.

Cell Cycle Measurements

Cells were harvested at different days (see Table 4), washed in PBS and fixed with 0.5% paraformaldehyde for 5 min, RNAse treated (100 U/ml) and stained with propidium iodide (50 μg/ml) in PBS with 0.1% Triton X-100 (27). After further washing in PBS, 10000 cells were analyzed in a FACScan flow-cytometer, equipped with a 15 mW, 488 nm, air-cooled argon laser. Linear FSC and red fluorescence (band pass filter >650 nm) were used. Data was converted to CONSORT 30 and analyzed with the sum of broadened rectangles model to obtain numbers and percentages of cells in different cell cycle phases.

IgM Secretion

Supernatants were harvested and stored at −20°C until tested with an ELISA assay. Ninety-six-microwell plates were coated with 200 μl/well of rabbit anti-human IgM (Fcμ, Pharmacia, Uppsala, Sweden) diluted 1 : 2000 in cold 0.1 M Na_2CO_3 at pH 9.5 overnight. The wells were further washed with a 0.9% NaCl, solution containing 0.05% Tween 20, before adding 100 μl/well of the standard or test samples diluted in PBS with 0.05% Tween 20. The plate was incubated for 2 h on a plate shaker, then washed and 100 μl/well of peroxidase-conjugated rabbit immunoglobulin anti-human IgM (μ chains) diluted 1 : 1000 in PBS with 0.05% Tween 20 were added. After further incubation and washing, an OPD solution in sodium citrate with H_2O_2 was added as substrate. The reaction was stopped after 6 min with 100 μl/well of H_2SO_4. Absorbance was measured at 492 nm in a spectrophotometer.

EBNA-1 Staining

2 × 10^6 cells, stimulated and harvested at day 4, washed in BSS were utilized to prepare smears, fixed in methanol/acetone (1/2, v/v) for 5 min, stored at 4°C overnight and stained with anti EBNA-1 serum, C' and anti-C' serum. At least 500 cells were counted using a fluorescence microscope at a magnification of 500 ×. Raji cells were used as a positive control (28).

Statistical Analysis

Mean values of groups were compared using Student's *t*-test. Stimulation indices were compared using the Wilcoxon's signed rank test.

RESULTS

Isolation of B-CLL Cells

Sixteen of 34 B-CLL patients had non-progressive disease at test. Ten of these 16 patients were in clinical stage 0, a condition also classified as monoclonal B lymphocytosis of undetermined significance (MLUS) (1,25). Leukemic cells from blood were isolated. Almost all cells (98%) expressed CD5, a hall-mark for B-CLL (3) (Table 2). Leukemic cells also expressed the pan-B molecule CD19. The IL-2 receptor was variably exhibited on the leukemic cells and some of these cells demonstrated a high-staining intensity for surface IgM (data not shown). No cells expressed the macrophage marker Mac-387.

In Vitro *Proliferative Response to SAC, IL-2 and BCGF*

Isolated leukemic cells were stimulated with SAC, IL-2, BCGF, and combinations of SAC plus IL-2, and SAC plus BCGF, respectively, for 3 days and analyzed for DNA synthesis (Table 3). Only the leukemic cell population from one of the patients in the non-progressive group demonstrated a prollferative but low response (L 150). In the group of patients with progressive

Table 2 Phenotypic Characteristics of Leukemic Blood Cells of B-CLL Patients in Relation to Disease Activity

Disease Activity	CD5 (%)	CD19 (%)	CD25 (%)	IgM (%)	MAC-387[a] (%)
Non-progressive disease. Stage 0 (n = 7)	93.0 ± 3.3[b,c]	96.0 ± 3.1	44.6 ± 18.6[d]	55 ± 20	0.6 ± 1.0
Non-progressive disease. Stage I (n = 4)	98.2 ± 0.5[c]	96.5 ± 1.3	51.2 ± 9.4[c]	30.3 ± 22.5[d]	0
Progressive disease (n = 13)	97.5 ± 2.5[c]	95.9 ± 4.1	47.2 ± 27.3[d]	30.8 ± 28.8[d]	0.3 ± 0.6
Normal B cells (n = 5)	29.2 ± 14.5	94.8 ± 4	18.2 ± 8.1	69.2 ± 10.0	0.8 ± 1.1

[a]Intra-cytoplasmatic antigen.
[b]Mean ± SD comparing normal B cells.
[c]$p < 0.001$ comparing normal B cells.
[d]$p < 0.05$ comparing normal B cells.

Table 3 Proliferative Response of Leukemic and Normal B Cells to SAC, IL-2 and BCGF

Patient Code	c.p.m. of Unstimulated Cells	IL-2	BCGF	SAC	SAC + IL-2	SAC + BCGF	Classification of Responder Status*
		(Stimulation Index)					
L 45	1144	1	5	1	5	7	−
L 63	90	3	3	3	7	4	−
L 65	105	1	1	4	10	4	−
L 71	2367	1	1	1	1	1	−
L 84	746	1	2	2	15	15	−
L 86	249	1	1	1	1	1	−
L 140	922	2	1	1	1	1	−
L 167	1268	1	2	1	1	2	−
L 172	1741	1	1	1	1	1	−
L 174	365	7	7	12	10	1	−
L 82	2869	2	1	1	1	1	−
L 150	331	2	47	3	36	50	+
L 159	1959	1	2	1	1	2	−
L 164	2052	1	2	1	1	3	−
L 170	593	1	1	1	3	1	−
L 173	589	1	4	1	5	6	−
L 50	294	6	4	5	3	2	−
L 68	692	4	2	175	262	29	++++
L 73	1307	1	2	100	88	94	++
L 160	770	1	6	31	42	63	++
L 169	1869	1	1	1	1	1	−
L 62	112	2	4	423	606	1955	++++
L 133	1046	1	4	1	5	5	−
L 141	276	2	20	26	241	30	++++
L 153	690	2	1	2	1	1	−
L 156	816	1	1	2	3	1	−
L 87	1157	1	2	53	41	9	++
L 161	3425	11	1	10	22	6	+
L 162	688	1	2	50	51	80	++
L 19	68	1	1	7	9	64	++
L 61	83	1	3	2	2	7	−
L 69	1964	1	1	1	1	1	−
L 70	370	7	2	4	7	1	−
L 158	779	4	3	90	214	345	++++
Normal unfractionated B cells ($n=3$)	2149 ± 2276	5	134	43	125	236	++++
High buoyant density normal B cells ($n=3$)	2433 ± 1393	1	1	1	2	5	−

*Scored as − (SI <20), + (SI 20–50), ++ (SI 51–100), +++ (SI 101–200), ++++ (SI >200).

disease, 10/18 patients had cell populations (56%) which responded to the stimulation agents, in some cases with high SI values. All responding patients had a proliferative response to SAC alone. The responses were often more pronounced in the presence of IL-2 and/or low MW BCGF (L 68, L 160, L 62, L 141, L 161, L 162, L 19, and L 158). The difference in response to SAC stimulation, alone or in combination, was statistically significant comparing progressive and non-progressive patients ($p < 0.05$).

In six cases (L 160, L 62, L 141, L 161, L 162, and L 158), the responding cells were analyzed for CD3, CD5, CD19, and CD21 expression after activation with SAC plus IL-2 (Figure 1). No cells expressed CD3. Most cells had high expression of CD5 and CD19 and a low expression of CD21. Thus, the responding cells seemed to be derived from leukemic B-CLL cells and not from contaminating normal cells.

In some cases (L 160, L 169, L 62, L 141, L 153, L 156, L 161 and L 158), stimulated and unstimulated B-CLL cells were also analyzed by changes in cell numbers, cell cycle positions, IgM secretion, and thymidine incorporation (Table 4). The responses were heterogeneous. In some non-responders (low DNA-synthesis rate; L 169 and L 153), the cells could still differentiate and secrete IgM. Populations measured as an augmented SI-value showed also, in some cases, an increase in total cell numbers and secreted IgM (L 160, L 141, and L 161), while in others (L 62 and L 158) only the cell numbers were increased but no IgM secretion was detected.

The expression of the EBNA-1 antigen was examined in long-term culture of responder cells of patients L 160 and L 158, to exclude that the proliferative response was dependent on EBV transformation. Leukemia B cells and normal unfractionated tonsillar B cells were negative for EBNA-1 after 4 days of culture (Table 4, data not shown).

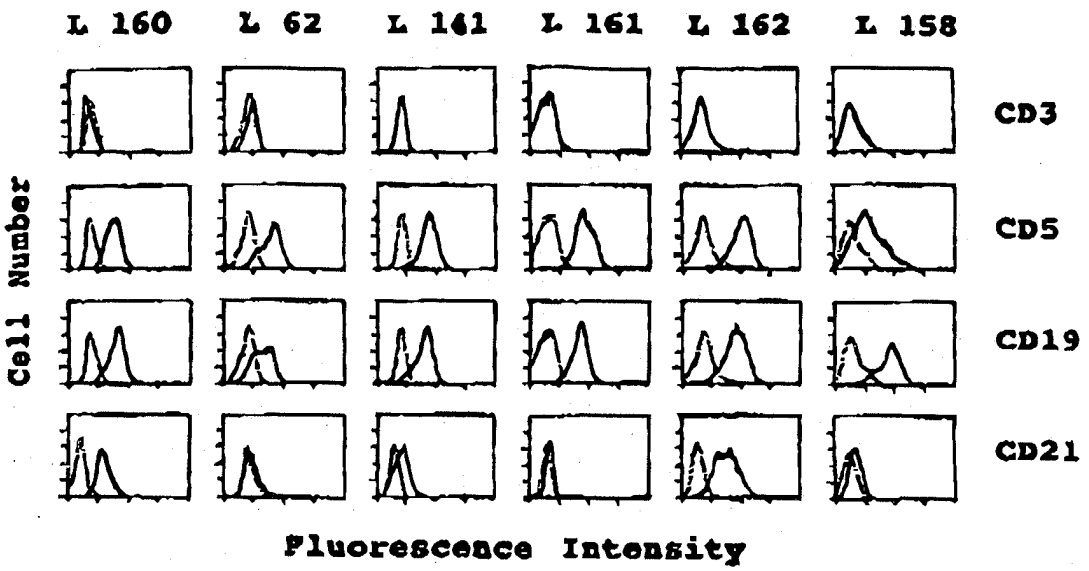

Figure 1 Cells from progressive B-CLL patients were stimulated with SAC + IL-2, harvested at day 4 and stained with antibodies (CD3, CD5, CD19, CD21). Dotted lines represent the background staining and solid lines the specific staining

Table 4 Proliferation and Differentiation in Leukemic B Cells Induced by SAC plus IL-2 Stimulation

Patient Code	Stimulatory Agents[a]	Time[b] (days)	DNA Synthesis[c] (c.p.m.)	Cell		Cell Cycle			IgM Secretion (ng/ml)
				Number ($\times 10^6$)	Viability (%)	G1 (%)	S (%)	G2 + M (%)	
L 160	−	0		10.0	100				
L 160	−	4	549	7.0	73				
L 160	+	4	48253	12.0	96	51	44	5	75
L 160	+	8	119258	12.0	90	38	51	10	95
L 160	+	12	36737	9.0	75	75	23	2	170
L 160	+	20	1586	1.0	44	76	14	10	210
L 169	−	0		10.0	100				
L 169	+	4	3111	8.5	47	5	94	1	55
L 62	−	0		10.0	100	97	2	1	
L 62	+	4	76700	16.0	62	7	86	7	0
L 141	−	0		10.0	100	98	1	1	
L 141	+	4	116612	12.5	83	23	72	4	1300
L 153	−	0		10.0	100				
L 153	−	4	1046	4.3	63				0
L 153	+	4	1578	9.0	45				16
L 156	−	0		10.0	100				
L 156	+	4	786	8.4	73				
L 161	−	0		10.0	100				
L 161	−	4	4353	7.6	82				14
L 161	+	4	27888	10.6	66				305
L 158	−	0		10.0	100				
L 158	−	4	862	8.0	83				0
L 158	+	4	47509	15.0	98	69	11	20	0
L 158	+	8	84768	21.0	70	11	14	75	0
L 158	+	12	121600	27.0	67	71	17	11	0
L 158	+	20	15629	18.0	46	70	19	11	0
NB	−	0		10.0	100	66	14	21	
NB	+	4	149396	16.0	62	28	65	7	50

NB, Normal unfractionated tonsillar B cells.
[a]Leukemic B cells from patients with progressive disease were cultured in the absence (−) or presence (+) of SAC plus IL-2.
[b]Time for measuring of DNA synthesis after initiation of culture. [c]^{3}H-thymidine incorporation.

DISCUSSION

Earlier studies have demonstrated that leukemic B-CLL cells might respond by DNA synthesis to a number of different signals (29). The present study extends those findings, showing a clear relationship between SAC-induced DNA synthesis and disease progression. Stimulated leukemic B cells may also differentiate. Although almost all of the responding leukemic B cells derived from the group of patients with progressive B-CLL, about half of the progressive patients had cells not responding to the stimulatory agents. The non-responsive leukemic cells may proliferate in response to other signals not used in the present study, or may show a delayed kinetic in their responsiveness (30).

Progressive B-CLL cells responded similarly as normal activated B cells with regard to SAC, SAC plus IL-2, and SAC plus BCGF stimulation. However, normal activated B cells also responded to BCGF alone, but not CLL B cells. The commercial BCGF preparation used in the present study is known to be contaminated with small amounts of other cytokines (31) which may influence the results. Fournier *et al.* (32) reported recently that recombinant low MW BCGF might stimulate B-CLL to proliferate and that this factor was also produced by the leukemic cells. The various results may be due to differences in the concentrations of BCGF and/or stimulation time. The expression of activation molecules between CLL populations varies a lot (15,33). Thus, cell populations with a certain pattern of activation molecules might be more prone to respond to BCGF, which may also explain the differences.

The high proliferative response of B-CLL cells from some patients with progressive disease may also be due to an aberrant oncogene expression, chromosomal translocations, or other generic abnormalities that accumulate during disease progression (3,34). An increased c-myc expression has been shown to prime B-CLL cells to self-renewal, but inhibits differentiation (35). The bcl-2 oncogene product favours B-cell survival, both *in vitro* and *in vivo* in transgenic mice, but not proliferation and differentiation (36,37). A loss of tumor suppressor gene activity has been described in B-CLL, such as low expression of the RB protein (38) and p53 mutations (39). With regard to p53, only a small number of B-CLL patients have been found to contain mutations at 'hot spots' defined in solid tumors (40). However, in a much larger proportion of progressive B-CLL patients, increased cellular levels and an altered configuration of p53 may be found, using the monoclonal antibody PAb 240 in an ELISA assay (44). This finding may indicate either a post-transcriptional modification of the protein, leading to a functional alteration similar to mutation, or a compensatory increase in the wild-type p53 as a consequence of a block in later parts of the p53 pathway (41,42).

Whatever the reason for the high proliferative response in progressive B-CLL cells to SAC, it may be considered in terms of signaling, especially as it has earlier been proposed that B-CLL cells may be under the regulation of normal T cells (14,16). A B-cell response can either be T-cell independent or dependent, and responding B cells may either self-renew or undergo terminal differentiation (33,43). It might be speculated that a high self-renewing capacity may relate to a rapid disease progression. In our material, patient L 156 (Table 4) may be an example of this. Several factors may contribute to the response of leukemic B cells. One of these can be signaling through surface Ig, by autoantigens, or peptides presented by MHC II molecules to CD4 T cells. Responding T cells may provide growth signals to the tumor clone. Other alterations, such as genetic or functional aberrations in the responding clonal B-CLL population may further add to the growth potential of leukemic cells. Studies are needed to explore such factors and to identify those of importance for the disease progression.

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REVIEW

Gerold Meinhardt · Clemens-M. Wendtner
Michael Hallek

Molecular pathogenesis of chronic lymphocytic leukemia: factors and signaling pathways regulating cell growth and survival

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Abstract Chronic lymphocytic leukemia is a malignant disease characterized by clonal expansion of relatively mature B-lymphocytes with a high percentage of cells arrested in the nonproliferative G_0/G_1 cell cycle phase. Possibly reflecting the clinical heterogeneity observed in patients, various signaling pathways may become affected during the initiation and course of this disease. This review discusses frequent alterations concerning proliferative, differentiation-inducing, and apoptotic pathways elucidated in the recent years that have improved our understanding of this disease.

Key words Chronic lymphocytic leukemia · Apoptosis · Cell growth · Signaling · Review

Abbreviations *BCR* B cell receptor · *bFGF* Basic fibroblast growth factor · *cAMP* Cyclic 3':5'-adenosine monophosphate · *CLL* Chronic lymphocytic leukemia · *ICE* IL-1β-Converting enzyme · *IFN* Interferon · *IκB* NF-κB inhibitor · *IL* Interleukin · *NF* Nuclear factor · *NF-ATp* Preformed NF of activated T cells · *PCD* Programmed cell death · *PTK* Protein tyrosine kinases · *sIg* Surface immunoglobulins · *STAT* Signal transducer and activator of transcription · *TGF-β* Transforming growth factor-β

GEROLD MEINHARDT studied medicine at the University of Munich, Germany, and subsequently pursued residency training in gastroenterology, nephrology, and hematology/oncology. He has also engaged in research at the Dana-Farber Cancer Institute in Boston, USA. He is presently a Fellow in hematology/oncology and heads a research group working on differentiation and apoptosis at the University of Munich.

MICHAEL HALLEK pursued his medical studies at the Universities of Regensburg and Munich, Germany, and Paris, France. He subsequently engaged in postdoctoral research in pharmacology and molecular biology, as well as training in internal medicine, hematology, and oncology. He currently heads the Laboratory of Gene Therapy of Hematopoietic Malignancies at the Gene Center and is a consultant in Hematology at the University of Munich.

Communicated by: Bertold Emmerich and Michael Hallek

G. Meinhardt · C.-M. Wendtner · M. Hallek (✉)
Medizinische Klinik, Klinikum Innenstadt,
Ludwig-Maximilians-Universität, Ziemssenstrasse 1,
D-80336 Munich, Germany

Introduction

Chronic lymphocytic leukemia (CLL) is a clonal B cell neoplasma of morphologically mature, functionally immature B cells expressing surface immunoglobulins (sIg). It is usually characterized as an accumulation of malignant B cells with low proliferative activity which escape the fate of programmed cell death (PCD; apoptosis) by a variety of mechanisms (see below). A variety of chromosomal aberrations have been described which are reviewed in a different contribution to this issue of the *Journal of Molecular Medicine* (Döhner et al.) It is unknown which, if any, of these chromosomal alterations is the initial transforming event in CLL. By an unknown mechanism, molecular defects are likely to induce the constitutive activation of several transmembrane signaling pathways which regulate the differentiation, cell cycle progression, and apoptosis of B cells (Tables 1, 2). From this point of view, CLL represents an attractive model for the study of defective signaling pathways in B cell neoplasms (Fig. 1).

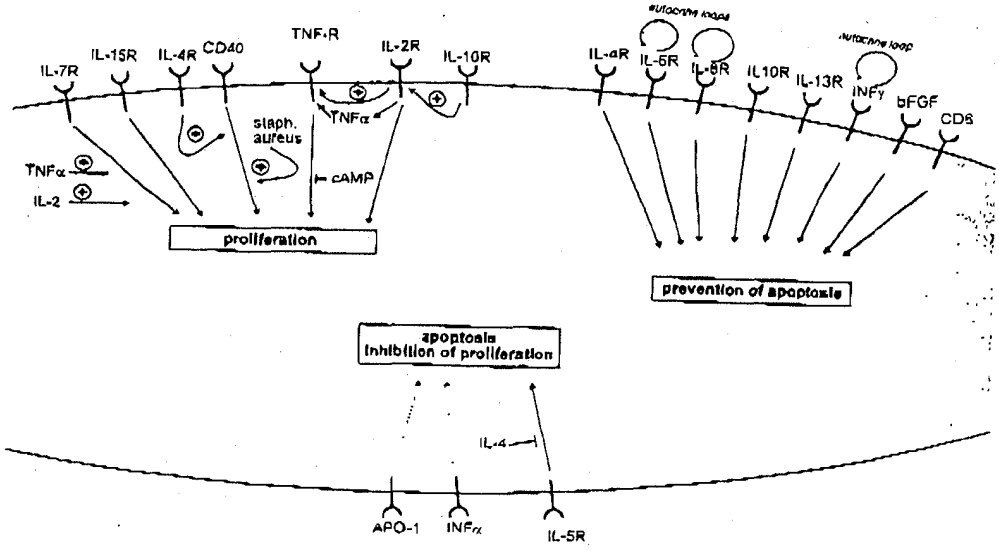

Fig. 1 Receptor-mediated responses in B-CLL cells

Factors regulating cell growth and survival of CLL cells

Cytokines elicit various responses in both normal and malignant lymphocytes that contribute to the regulation of survival, differentiation, and apoptosis.

Factors promoting cell growth

One of the most important growth factors in CLL is *tumor necrosis factor-α* (TNF-α) [1, 2]. This growth-promoting action of TNF-α is counterbalanced by the second messenger cyclic 3':5'-adenosine monophosphate (cAMP) [3]. In comparison to B-monoclonal lymphocytosis of undetermined significance, CLL cells show a higher proliferative response to the combined stimulation with anti-*CD40* monoclonal antibodies and *Staphylococcus aureus* [4]. In addition to a proliferative stimulus via the CD40 receptor, costimulatory signals such as the B7 antigen are activated by agonistic CD40 monoclonal antibodies or CD40 ligand molecules in CLL [5]. Cross-linking with monoclonal anti-CD40 antibodies in combination with interleukin (IL) 4 is also able to induce entry of CLL cells into cell cycle, while differentiation into immunoglobulin secreting B cells is not enhanced.

IL-2 has the potential to induce IgM synthesis in a synergistic manner with *S. aureus* strain Cowan I [6]. Furthermore, IL-2 upregulates the expression of the TNF receptor and secretion of TNF-α, thus rendering CLL cells more susceptible to the growth promoting effects of this cytokine, possibly in an autocrine loop [7]. Interestingly, IL-2 receptor expression is upregulated in response to IL-10 treatment, while IL-10 alone has been shown to induce apoptosis in CLL cells [8].

IL-7 can induce proliferation of both CLL cells and cells from acute leukemias. This stimulatory effect seems to be potentiated by IL-2 and TNF-α [9].

IL-15 has the potential to trigger both proliferation and immunoglobulin synthesis [10]. In distinction to normal B-lymphocytes, CLL cells elicit this in vitro effect without preactivation with mitogens or other stimuli. The proliferation to IL-15 is dependent on the β (p75) and γ (p64) chains of the IL-2 receptor, while the specific α chain which distinguishes IL-2 and IL-15 receptors seems to be less relevant for this function.

Factors preventing apoptosis

IL-4 is a T cell derived multifunctional cytokine which protects CLL cells from apoptosis without a significant effect on proliferation. This effect may be due to greater expression of IL-4 receptors on CLL cells than on normal cells [11]. Interestingly, it has been reported that the protective effect of IL-4 against spontaneous apoptosis is significant only in cells from previously treated CLL patients, and is nearly absent in CLL cells from untreated patients [12]. While IL-4 has only a modest effect upon the proliferative response of CLL cells, it profoundly inhibits the proliferative response to TNF-α, INF-α, and IL-2 [13].

IL-6 is a pleiotropic cytokine influencing antigen-specific immune responses and inflammatory reactions and is an important B cell differentiation factor and T cell activation factor. IL-6 is also the predominant regulator of myeloma cell growth. CLL cells are protected from spontaneous apoptosis in the presence of IL-6. Moreover, CLL cells are capable of secreting IL-6 protein, which is further enhanced in the presence of TNF. Increased serum IL-6 levels have been detected in 27 of 50 cases of CLL, the mean level being significantly higher in advanced stages of disease [14]. This suggests a potentially relevant autocrine loop at least in some patients with progressive disease [15].

IL-8 is a chemoattractant protein that can specifically activate neutrophil granulocytes. A constitutive expression of both IL-8 mRNA and IL-8 secretion is found in CLL [16]. IL-8 prolongs the survival of resting CLL cells in

Table 1 Differential effects of selected factors on normal B and on B-CLL cells. (*A* Apoptosis, *D* differentiation, *P* proliferation – usually requiring preactivation)

	B lymphocytes	B-CLL
IL-2	*P* in the presence of additional factors	*P*
IL-4	*P* + *D*	Protection from *A* (possibly due to increased IL-4R expression)
IL-5	*D* (*P*)	*A*?
IL-6	*D*	Protection from *A*
IL-7	*P* of pre-/pro-B cells, no effect on mature B cells	*P*
IL-8	Chemokinetic response, inhibition of IL-4 induced *P*	Protection from *A*
IL-10	*P* + *D*, prevention of *A* of germinal center cells, promotion of *A* in later stages of differentiation	*P*, protection from *A*
IL-13	*P* + *D*	Protection from *A*
IL-15	*P*, IgG synthesis after preactivation	IgG synthesis without preactivation
Anti-CD6	–	Protection from *A*
Anti-CD40	*P* + *D*, inhibition of *A*	*P*
INF-α	Differential (dose-dependent), effects on proliferation	Differential in vivo and in vitro effects
INF-γ	*P*	Protection from *A*
TNFα	*P* + *D*, chemokinetic response	*P*
bFGF	*P*	Protection from *A*
TGF-β	Inhibition of progenitor *P*	Inhibition of *P*
APO-1/ FAS/CD95	*A*	(Downregulated receptor)

Table 2 Oncogene products of potential relevance for the pathogenesis of CLL

Oncogene products involved in growth-promoting pathways	Oncogene products involved in anti-apoptotic pathways
Protein tyrosine kinase pathways	Bcl-2/Bax
Ras	p53/MDM2
Cyclin D_2	

most of the cases studied. The dose range of IL-8 used in this experiment is comparable to the levels of IL-8 released by CLL cells, indicating that the anti-apoptotic action is exerted at physiological doses. After antibody-mediated neutralization of endogenous IL-8, increased numbers of apoptotic cells are detected, suggesting that IL-8 is another auto- or paracrine survival factor for CLL [16].

IL-10 induces growth and most notably differentiation of normal human B-lymphocytes into immunoglobulin secreting cells and prevents apoptosis of germinal center B cells [17]. In B-CLL cells IL-10 has been shown to promote DNA synthesis and IgM production when cells are preactivated with anti-CD40 antibodies [8]. IL-10 synergizes with IL-2 for proliferation and differentiation by increasing high-affinity IL-2 receptors, a phenomenon seen on both normal and leukemic B cells. In contrast, in nonactivated B-CLL cells IL-10 mediated apoptosis in a dose-dependent way [17]. This apoptotic effect is counteracted and overcome by T cell derived cytokines [IL-2, IL-4, interferon (IFN)-γ] and anti-CD40 stimulation. In normal human B cells the effects of IL-10 have been shown to differ depending on the state of activation, while during the initial activation of the Ag receptor IL-10 promotes apoptosis and in a later state differentiation [18]. Meanwhile there is convincing evidence that IL-10 promotes cell survival in B-CLL as has been demonstrated in experiments with fresh tumor samples of CLL patients (in contrast to frozen samples with reduced IL-10 binding specificities, an apoptotic effect was not seen) [19]. This observation is further supported by common signal transduction events with IFNs which are known to protect CLL cells from PCD.

IL-13 is a cytokine produced by TH2 helper T cell clones following activation. There is considerable overlap concerning the biological activities of IL-4 and IL-13, especially with regard to B cells. IL-13, in contrast to IL-4, has no proliferative effect on activated T cells. IL-13 stimulates the growth of normal B cells, however, fails to have a similar effect on CLL cells. Furthermore, IL-13 protects CLL cells from in vitro spontaneous apoptosis. The effects of IL-13 on neoplastic B cells are slightly less pronounced than those of IL-4 and occur independently of the presence of IL-4. Whereas IL-2 induces proliferation of CLL cells, addition of IL-13 abrogates this effect [20].

IFN-γ inhibits PCD of CLL cells in vitro. Moreover, serum samples of CLL patients show increased serum levels of IFN-γ. Since high levels of IFN-γ mRNA are detected in T cell depleted CLL blood samples, and CLL cells contain measurable levels of IFN-γ receptor, it has been postulated that an autocrine IFN-γ loop contributes to delaying apoptosis [21].

Basic fibroblast growth factor (bFGF) is a pleiotropic cytokine which seems to delay fludarabine-induced apoptosis in CLL cells capable of producing augmented amounts of bFGF [22].

The *CD6* antigen is a membrane glycoprotein and part of a large family of proteins, including CD5 and type I macrophage scavenger receptor. It binds to the activated leukocyte cell adhesion molecule and is an important costimulatory molecule on T lymphocytes. CD6 is found predominantly on mature T cells, thymocytes, and a small subpopulation of normal B cells. In contrast, most CLL

cells are positive for CD6. Anti-CD6 stimulation protects against anti-IgM induced apoptosis in CLL [23].

Factors stimulating apoptosis

IL-5 is a hematopoietic growth factor acting primarily on eosinophils. The high-and low-affinity receptors for IL-5 can be found on all hematopoietic and lymphoid cells. The action of IL-5 regarding CLL cells is somewhat controversial at the moment. Prentice and coworkers [24] reported that IL-5 increased spontaneous apoptosis of CLL cells by 53% after 2 days in culture in all 13 cases studied. This effect was specific for CLL cells, since it was not observed in normal peripheral blood B cells. The pro-apoptotic effect of IL-5 was counterbalanced by addition of IL-4.

APO-1/Fas antigen (CD95) is as a surface molecule capable of transducing apoptotic signals into cells. APO-1/Fas antigen is detectable on the surface of many cell lines of myeloid and lymphoid lineage, including normal B cells. In contrast, little or no Fas antigen expression is seen in CLL cells [25]. Thus CLL cells may acquire resistance to the apoptotic pathway stimulated by the Fas ligand by down-regulation of the Fas antigen.

Factors with miscellaneous or contradictory effects

IFN-α is capable of inhibiting the growth of various tumor types in vitro and can inhibit especially the proliferation of CLL cells in a subpopulation of patients which has been the subject of a number of clinical trials [26–28]. In the majority of patients IFN-α protects CLL cells from PCD in vitro [29]. These conflicting in vivo and in vitro results could be explained by additional regulatory effects that IFN-α has on other cell types in vivo. Therefore IFN-α may reduce the number of malignant cells by interfering with autocrine growth factor loops [30]. Thus in a given patient the observed in vitro effects of selective administration of IFN-α might differ from the results in a therapeutic setting and need to be interpreted with caution.

CLL and normal B cells express the receptors for the transforming growth factor-β (TGF-β). CLL cell proliferation is variably inhibited with addition of TGF-β. Interestingly, the level of TGF-β inhibition is lower in CLL cells than that observed in normal B lymphocytes. Furthermore, the lower sensitivity of leukemic B cells to TGF-β is in accord with decreased expression of the TGF-β receptors, thus underlining the proliferative advantage for the CLL cells [31]. This is further augmented by an increased TGF-β (and decreased IL-6) production by leukemic cells and stromal cells in CLL patients, resulting in suppression of normal bone marrow cells and expansion of the leukemic B cell clone [32]. In addition, CLL cells show resistance towards the apoptosis-inducing effects of TGF-β, whereas normal B cells exhibit increased apoptosis upon TGF-β stimulation [11].

Intracellular signaling

Signals promoting cell growth

B cell receptor signaling

CLL cells are characterized by the expression of the CD5 antigen and membrane immunoglobulin of IgM or combined IgM and IgD subtype. The membrane Igs are noncovalently associated with a Igα/Igβ heterodimer, also called CD79a and CD79b, respectively, thus forming the functional B cell receptor (BCR) [33]. The CD79a/b heterodimer seems to be essential for the coupling of mIg to intracellular signaling events resulting in cell cycle progression [34]. Activation of some nonreceptor *protein tyrosine kinases* (PTK) by the BCR is of central importance for its biological effects in B cells: the Src-PTK (Lyn, Fyn, Blk, Lck) and Syk [35–41]. Signal transduction of the BCR is mediated by the immunoreceptor tyrosine-based activation motif in the cytoplasmic domains of the B29/mb-1 heterodimer that are phosphorylated on critical tyrosine residues resulting in activation of Src and Syk family kinases [34, 42]. In CLL cells BCR ligation usually results in an increase in intracellular calcium and in cell division. This calcium release is decreased in a subset of CLL patients expressing marked levels of CD11b antigen, a leukocyte adhesion molecule belonging to the integrin superfamily [43]. This defective calcium release is associated with changes in the pattern of protein tyrosine phosphorylation of cytosolic phosphoproteins, including the *phospholipase C-γ_1*. Thus, the *phosphoinositide pathway* may be disrupted in this subset of CLL at an early step between surface Ig receptors and protein tyrosine kinases. Whether phospholipase C-γ_2 which is predominantly expressed and tyrosine-phosphorylated upon stimulation in normal B lymphocytes is also affected in CLL is unknown [44]. A possible explanation is a defect in *G proteins* which block signaling from sIg to phospholipase C, resulting in decreased production of the second messengers inositol 1,4,5-triphosphate and diacylglycerol, thus preventing activation of the serine/threonine protein kinase C [45]. Alternatively, the phosphoinositide pathway might be disrupted by an altered expression of tyrosine phosphatases such as CD45 [46].

CLL cells from a subgroup of patients are unable to proliferate after stimulation of the BCR. In this subgroup a decreased activity of the BCR-associated PTK complex and a diminished expression of *Syk* is detected, while other PTKs such as Lyn, Blk, Btk, Lck, and Csk are expressed at similar levels as the responsive subtype [47]. Furthermore, the *Shc/Grb2* complex as a crucial signal transducer in a tyrosine kinase cascade is not formed in this subtype of CLL, and consecutive activation steps including the phosphorylation of *p21^{ras}* are impaired. This suggests that the *Ras pathway* is important in CLL, similar to the situation in multiple myeloma. These observations are of potential clinical relevance because BCR-mediated mitogenic responses are correlated with clinical disease progression in

CLL [48]. In this context it seems important that a subgroup of CLL which is nonresponsive to IgM antibodies is negative for the surface marker CD38 while the subgroup responding to BCR stimulation by rapid calcium mobilization and apoptosis is CD38 positive [49].

Recently Thompson and colleagues [50] showed that the loss of surface BCR and defective BCR signaling characteristic of CLL can be deduced to an aberrant B29 (CD79b and Igβ) expression, which was detected in more than 80% of examined CLL samples. Half of these patients had highly reduced B29 mRNA levels, and the rest showed mutations on the cDNA level, such as point mutations, insertions, and deletions. The somatic mutations were located largely in the B29 transmembrane or cytoplasmic domains; the exact effects of these mutations on the signaling cascade are currently under investigation. In summary, aberrant B29 expression leads to loss of surface BCR expression and defective signaling, contributing probably to prolonged survival and resistance to apoptosis.

Signaling further downstream towards the nucleus via various *transcription factors* has been elucidated in particular by studying the effects of TNF-α, which is one of the strongest growth factors in CLL. TNF-α signaling is mediated by activation of nuclear factor (NF)-κB and the transcription factor family AP-1 [51]. The activation of the *AP-1* complex, which is composed of c-Jun and c-Fos proteins, is regulated quite differentially. On the one hand, signals from growth factor receptors converge via the Ras/extracellular signal regulated kinase pathway at a major regulatory element of the promoters of the *c-fos* and other genes, the serum response element, targeting the TCF proteins Elk-1 and SAP-1 [52]. The other transcription factor, c-Jun, is regulated by a family of mitogen-activated protein kinases distinct from the extracellular signal regulated kinases, i.e., the Jun N-terminal/stress-activated protein kinases, which are activated by extracellular signals [53]. Interestingly, in CLL the two components of AP-1, i.e., c-Jun and c-Fos proteins, are not generated in response to TNF. Several defects in the induction of c-Jun and c-Fos have been found: missing nuclear translocation, total absence of c-Jun and c-Fos in the cytoplasm or the nucleus, and the induction of only c-Fos (preventing the functional heterodimerization with c-Jun). In contrast, the levels of JunD, c-Fos, and Fos-B as members of the AP-1 factor family are found to be higher in nonstimulated B-CLL than in normal B lymphocytes. It has been speculated that extracellular signal regulated kinases or Jun N-terminal/stress-activated protein kinases are activated in the unstimulated malignant cell clone [54].

NF-κB seems to be induced by TNF via the reactive oxygen intermediate pathway: NF-κB activation is abolished by pretreatment of CLL cells with antioxidants [51, 55]. However, the reactive oxygen intermediates involved in coupling TNF receptor stimulation to NF-κB need to be defined. It has been shown that activation of NF-κB by TNF is associated with phosphorylation and degradation of the NF-κB inhibitor (IκB) α, which occurs independently of protein kinase C stimulation, while calcium-dependent pathways synergize with TNF-α at the level of IκB degradation [56]. An important mediator of Ca^{2+}-dependent events is calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase, and it is known that calcineurin can be activated via the CD5 receptor molecule, which is also abundant in CLL [57, 58]. TNF-α induced IκBα phosphorylation (and consecutive degradation resulting in NF-κB activation) is dependent on IκBα protein kinases since IκBa degradation can be blocked by nonspecific protease inhibitors [56]. Once IκBα phosporylation has been triggered, Ca^{2+}-dependent but calcineurin-independent pathways may enhance IκBα proteolysis, the Ca^{2+}-dependent protease calpain being one candidate [59, 60]. Taken together, this suggests that some defects in NF-κB activation involving IκBα protein kinases and proteases exist in CLL. This is underscored by the observation that NF-κB2/p52, a member of the Rel/NF-κB family, is constitutively present in the nucleus of unstimulated CLL cells. This could represent a critical preactivation status of CLL since it is known that binding of Bcl-3 to NF-κB2/p52 homodimers results in further transcriptional activation [61].

Other transcription factors such as *preformed NF of activated T cells* (NF-ATp), *NF-κB2/p52*, and several AP-1 family members seem to be constitutively translocated to the nucleus and bound to DNA in CLL cells [54]. In normal B lymphocytes NF-ATp is located in the cytoplasm and is activated by CD40 ligand and IL-4 or by calcium-/calmodulin-dependent dephosphorylation by calcineurin, resulting in nuclear translocation [62, 63]. In CLL nuclear translocation of NF-AT might result from activation by the CD5 receptor since CD5 triggers intracellular calcium release and activates calcineurin [57]. It has not yet been defined whether this CD5-triggered calcium release is signaled by the PTK Lck in CLL [64, 65]. NF-κB2/p52 is also constitutively present in the nucleus of unstimulated CLL cells. This could represent a critical preactivation status of CLL since it is known that binding of Bcl-3 to NF-κB2/p52 homodimers results in further transcriptional activation [61].

Another hallmark of CLL involving dysregulation of transcription factors is the constitutive nuclear translocation of NF-ATp, a member of the family of NF-AT factor [54]. This phenomenon is seen in unstimulated B cells from CLL patients but not in normal B lymphocytes, where the nuclear translocation and activation of NF-AT factors are dependent on calcineurin or is induced by CD40 ligand and IL-4 [62, 63]. This argues that calcineurin-involved Ca^{2+} signaling pathways resulting in nuclear translocation of NF-ATp are preactivated in B-CLL. In this context it is, again, of note that activation of the CD5 receptor which is abundant in CLL triggers intracellular calcium release and stimulates calcineurin. It has not yet been determined whether this CD5-triggered calcium release is signaled by the PTK Lck which is known to play an important role in Ca^{2+}-mediated activation of Jurkat T cells [64, 65]. Interestingly, in malignant B cells (murine B cell lymphoma cell line A20) it has been shown that transcription does not require de novo protein synthesis after TNF-α stimulation, and that calcium flux alone is sufficient.

which is in contrast to the regulation of other genes, where NF-AT proteins play a role. Furthermore all TNF-α NF-AT binding sites are capable of binding NF-ATp in the absence of AP-1 [66].

β_2-*Adrenergic signaling*

Human lymphocytes bear β_2-adrenergic binding sites whose interaction with catecholamines results in the stimulation of the enzyme *adenylate cyclase* via the stimulating *GTP binding protein*, thereby increasing the intracellular concentration of cAMP [67]. This second messenger has been shown to enhance the differentiation of both immature lymphocytes and lymphoma cells [68]. Interestingly, plasma, urine, and intracellular cAMP levels are reported to be decreased in patients with CLL [69]. Both adrenergic binding sites and the stimulation of adenylate cyclase by catecholamines have been shown to be reduced in CLL lymphocytes [70, 71]. The density of both β_2-adrenergic receptors and cAMP production upon catecholamine stimulation decreases with disease progression from Binet stages A to C [72]. CLL cells showed a reduced responsiveness of adenylyl cyclase to forskolin, most likely caused by reduced availability of functional active α subunit of stimulatory G proteins [72, 73].

Signals preventing cell death

Apoptosis or PCD is a physiological process that plays a pivotal role in the regulation of tissue homeostasis. It is characterized morphologically by cell shrinkage, reorganization of the cell nucleus, active membrane blebbing, and fragmentation of the cell into membrane enclosed vesicles, termed apoptotic bodies [74–79].

Current two- or three-step models of apoptosis can be summarized as a sequence of events involving an initiating step, a cascade of intracellular signaling steps, and a final execution phase [80]. Events initiating apoptosis are the stimulation of specific receptors (Fas, TNF) [81, 82], DNA damage [83], ceramide generation [84], or growth factor withdrawal [85]. The intracellular signaling or amplifying cascade transduces the pro-apoptotic stimulus via a series of proteases towards one or several parallel downstream proteases that cleave vital substrates such as poly(ADP-ribose) polymerase [86], lamins [87], α-fodrin [88], actin [89], RB [90, 91], the 70-kDa protein component of the U1-ribonucleoprotein [92], the catalytic subunit of the DNA-dependent protein kinase [93], sterol regulatory element binding proteins [94], and protein kinases [95]. These signaling pathways can be modulated by crmA- [96] or p35-like inhibitors [97]. Furthermore, a delicate balance of homo- and heterodimers of Bcl-2 family proteins modulates the pro-apoptotic signal, either permitting or inhibiting the amplifying cascade and the final execution phase, including the release of cytochrome C from mitochondria and the generation of reactive oxygen species [75]. In the last phase, beyond a yet undefined point of no return, the activation of endonucleases, permeabilization of membranes and proteolytic processing of substrates takes place. This picture is complicated by various proteins, protein families or signaling pathways that can further modulate an apoptotic response at certain steps by activation of transcription (p53, cytokines), interfering with the cell cycle machinery (inhibitors of cyclin-dependent kinases, cytokines) or phosphorylation of important proteins such as Bcl-2 and Bad (serine/threonine kinases).

Caspases

Much evidence has accumulated over the past few years that proteases may be the key executioners of PCD. It is known from studies of the nematode *Caenorhabditis elegans* that a gene termed ced-3 is necessary for timely eliminating 131 out of 1089 cells during early morphogenesis [98]. The mammalian homologue of ced-3 is the IL-1β converting enzyme (ICE), a cysteine protease responsible for maturation of IL-1β by proteolytic cleavage of an inactive 33-kDa IL-1β proenzyme [99]. Several ICE-related proteins have been identified which belong to a continuously expanding family of ICE-like proteases, now termed caspases [100]. Overexpression or activation of each caspase is able to confer apoptosis to certain cell types. The activation of only a subset of these caspases is required in a cell to undergo apoptosis, because apoptotic responses can normally occur in null-mice lacking a particular caspase gene. Thus, different pro-apoptotic pathways may require only one or a few distinct members of the caspase family, depending on the stimulus [101]. The specific functions or physiological substrates of various caspases are still largely unknown. It is possible that caspases have important physiological roles beyond their pro-apoptotic function. Work on signaling involving the APO-1/Fas or TNF receptors has revealed that a coagulation-like cascade of proteases confers an apoptotic signal towards cytoplasmic downstream proteases. These executioner-proteases such as caspase-3 (CPP32/Yama/Apopain) cleave vital cellular proteins at specific sites (aspartates).

In CLL cells the proteolytic cleavage of the 116-kDa *poly(ADP-ribose) polymerase* has been studied in patient samples after incubation with dexamethasone and fludarabine with respect to spontaneous apoptosis. A 85-kDa cleavage product resulting from caspase-mediated proteolysis was detected in all samples. Moreover, incubation of CLL cells with Z-VAD.fmk, a specific inhibitor of members of the caspase family, blocked apoptosis in most cases [102]. These results suggest that CLL cells retain the capacity to activate caspases spontaneously and after chemotherapy. Further studies are needed, however, to determine the precise involvement of individual caspases and to search for defects regarding such activation and subsequent substrate degradation.

Bcl-2 protein family

The *bcl-2* gene was initially cloned from the t(14;18) translocation breakpoint found in low-grade follicular lymphoma [103]. Several Bcl-2 related proteins share three highly conserved regions and can variably form homo- and heterodimers, founding a family of cell death regulators, and suggesting that Bcl-2 functions, at least in part, through protein-protein interactions [75, 104, 105]. Overexpression of Bcl-2 has been implicated with resistance to apoptosis, whereas overexpression of Bcl-2 antagonists, such as Bax, Bad, Bid, and Bcl-x_s, is involved in inducing apoptosis [106–110]. This is complicated by the fact that other anti-apoptotic and dimerizing proteins such as Bcl-x_l and E1B-19K, Bag, Mcl1, A1, and Brag have recently been identified [111–115]. Moreover, the dimerization of Bcl-2 family proteins can be modulated by serine phosphorylation of at least two members, Bcl-2 and Bad [116, 117]. It is likely that a delicate balance of dimers, possibly involving additional, still unidentified proteins, regulates the further execution of cell death signals.

Bcl-2 protein is highly expressed in a variety of lymphomas. Lymphocytes of patients with CLL can have high amounts of Bcl-2 even in the absence of the t(14;18) translocation, resulting in a strong resistance towards corticosteroid-induced apoptosis [118]. The incidence of *bcl-2* gene rearrangements in CLL appears to be less than 5% [119]. The overexpression of Bcl-2 is associated mostly with low Apo-1/Fas Ag expression [120]. No statistically significant difference in bcl-2 protein (over-)expression is noted between cells of patients sensitive or resistant to chlorambucil, fludarabine, and 2-chlorodeoxyadenosine [121]. The cause for Bcl-2 overexpression in CLL cells may be caused, at least in part, by a complete demethylation of both copies of the *bcl-2* gene, as found in all 20 cases of CLL studied [119]. Hypomethylation of a gene often leads to upregulation of gene expression. In contrast, analysis of lymphoma cell lines that harbor a t(14; 18) showed hypomethylation of only one of the two *bcl-2* alleles in one of six patients studied and no demethylation in any of the five lymphoid cell lines examined. Since Bcl-2 protein levels were not always correlated with the methylation status, additional mechanisms are likely to contribute [119].

Various cytokines have been shown to regulate Bcl-2 or to depend upon Bcl-2 expression to elicit a certain cellular response. The promotion or prevention of the IL-10-mediated apoptosis has been found to be correlated with the expression of Bcl-2 [17, 18]. IL-8 incubation leads to upregulation of *bcl-2* mRNA [16], whereas bFGF upregulates Bcl-2 protein levels [122]. Conflicting results have been reported concerning the regulation of Bcl-2 in IL-4-treated CLL cells. Dancescu et al. [123] describe a significant induction of Bcl-2 protein in IL-4-treated CLL cells compared to unstimulated, hydrocortisone-treated, and fresh CLL cells, whereas Frankfurt et al. [12] found no change in Bcl-2 protein level. Whether these discrepancies merely reflect different experimental conditions needs to be determined.

A new class of chemotherapeutic drugs, the taxans, can induce apoptosis by affecting the integrity of microtubules. This prevention of polymerization or depolymerization of cellular microtubules is associated with phosphorylation of Bcl-2, whereas anticancer drugs merely damaging DNA do not. The Bcl-2 phosphorylation seems to depend upon Raf-1 activation [124]. Taxans are active in the G_2–M phase of the cell cycle, where the reorganization of microtubules predominantly occurs. Phosphorylation of *bcl-2* in drug-treated cancer cells seems to be restricted to this cell cycle phase as well. In accordance, CLL cells, which are blocked mostly at G_0–G_1, do not show Bcl-2 phosphorylation despite their relative overexpression of this protein [125].

Experiments with the topoisomerase I inhibitor 9-amino-20(s)-camptothecin, and the purine analogue fludarabine, have revealed a correlation between drug-induced apoptosis and the ratio of endogenous levels of Bcl-2 to *Bax*. None to low Bcl-2/Bax ratios are associated with drug-sensitivity, compared to CLL cells with intermediate to high ratios which are drug-resistant [126].

Bax upregulation and Bcl-2 downregulation are also found in anti-IgM- and glucocorticoid-induced apoptotic cells, respectively. Furthermore, *CD6* cross-linking downregulated bax mRNA levels in anti-IgM treated cells, resulting in an increased *bcl-2/bax* ratio. CD6 activation also prevents bcl-2 mRNA downregulation and apoptosis induced by glucocorticoid in one of six glucocorticoid-sensitive patients. Stimulation of CD6 might contribute to CLL survival by the modulation of the Bcl-2/Bax ratio [23].

p53

The p53 tumor suppressor gene is the most frequently mutated or deleted gene in human cancers. p53 encodes a transcriptional activator that is dispensable for normal development but is part of a crucial cellular damage control pathway. Overexpression of wild-type p53 into cells that have lost endogenous p53 induces apoptosis in a range of cultured cell systems [127, 128]. p53 has been termed "guardian of the genome" since its predominant function is to suppress outgrowth of genetically damaged, hence potentially neoplastic cells. p53 tetramers bind to DNA sequences in the promoters or introns of specific genes regulating growth. Such targets include $p21^{WAF1/CIP1}$ [129, 130], an inhibitor of the cyclin-dependent kinases, and GADD45 (growth arrest and DNA damage), a protein that can inhibit cell growth by itself when overexpressed [131]. Furthermore, p53 increases expression of the pro-apoptotic protein Bax in some but not all cell types [132, 133], the insulin-like growth factor-binding protein [134], and the apoptotic trigger Fas/Apo1 [135] and represses Bcl-2 [136]. Presently it is not clear how different degrees of DNA damage may target p53 function towards a cell cycle arrest or the induction of the apoptotic pathway. To further complicate the picture, p53 can interact with such important cellular targets such as E2F-1, retinoblastoma protein, Wilms tumor 1 protein 1, DP-1, and the Abl tyrosine kinase.

Numerous studies have attempted to quantify the extend of *p53 mutations* in various tumor entities. In B-CLL, mutations of the p53 gene are found in 10–17% of patients examined [121, 137–143]. A few studies have compared the response to chemotherapy with the p53 status. These data suggest a significantly higher risk of disease progression and death in patients with p53 mutations. In addition, the number of patients achieving at least partial remission is significantly lower with a preexisting p53 mutation [138, 139, 144]. Only one report found no association with clinical stage [140]. Resistance to chemotherapy is, however, not necessarily related to a p53 gene mutation [121]. A multivariate analysis has shown p53 gene deletion to be the strongest prognostic factor in the survival of patients treated with purine analogues [141]. p53 mutations predominate in advanced clinical stages with rather aggressive disease (Binet stage C) [145].

p53 function can be altered not only by mutation or gene (chromosome 17q) loss but also by binding to the product of the murine double minute-2 (*MDM2*) gene, thus inhibiting its ability to activate transcription [146]. In the absence of p53, mdm2 confers a growth advantage to cells and can overcome a G_1 cell-cycle arrest induced by p107, a member of the pRb tumor-suppressor family [147]. A recent study found 28% of patients with B-CLL or non-Hodgkin's lymphoma had more than tenfold higher levels of MDM2 gene expression than that observed in normal B cells. Moreover, MDM2 gene overexpression has been found more frequently in patients with the low-grade type of lymphoma and significantly more frequently in patients at advanced clinical stages [148]. Several distinct MDM2 proteins have been found in human cell lines. In 64% of B-CLL samples the MDM2 proteins p57, p59, p67, and p90 have been found to be overexpressed in various combinations compared with normal volunteers. The MDM2 protein p57 was predominantly overexpressed 53% in CLL [125]. Although this study did not detect a statistically significant correlation of MDM2 protein overexpression to clinical disease stage and history of previous chemotherapy, it did, however, suggest an important role for MDM2 in the early pathogenesis of CLL that needs further evaluation.

Cyclins

Cyclins are proteins being expressed and activated at certain stages in the cell cycle that, in conjunction with cyclin-dependent kinases and specific inhibitors, regulate the orderly progression through the cell cycle. Of the several distinct classes of mammalian cyclins identified so far, there is increasing evidence that D-type cyclins, which regulate the transition from G_0/G_1 to S phase, may play a significant role in establishing the malignant phenotype. It has been shown that *cyclin* D_2 mRNA is on average five- to tenfold overexpressed in most of the CLL samples studied, as compared with normal resting B lymphocytes in which cyclin D_2 mRNA is barely detectable [149]. Transfection experiments have suggested that cyclin D_2 may act more as a survival factor than a proliferation initiator. Growth factor withdrawal or induction of differentiation in certain cell types, normally resulting in PCD, does not induce apoptosis if these cells overexpress cyclin D_2 protein [150]. It has been speculated that CLL cells in early G_1 rather than in a G_0 phase retain their capacity to respond to external signals, and that this thus provides a survival advantage compared to quiescent cells [149].

STATs

Comparing factors which exert an anti-apoptotic effect on B-CLL as IFN-α, IFN-γ, IL-4, IL-6, and IL-10, it is striking that they all involve activation of members of the family of Janus kinase and signal transducer and activator of transcription (STAT) [52]. Janus kinases associate with the cytokine receptors which lack intrinsic kinase activity and activate the STATs as the transcription factor targets on specific tyrosine residues. The tyrosine phosphorylation of the STATs allows them to dimerize, translocate to the nucleus, and bind specific DNA sequences in the promoters of target genes. Functionally crucial is a differential STAT activation which results in specific DNA binding activities. In this context it is important that activation of the IL-10 receptor of B-CLL results in phosphorylation of STAT1 and STAT3 proteins, which is also seen for IFN-α, and is similar to IFN-γ (STAT1) [19]. Another study has demonstrated that the IL-10 receptor has similar signaling functions to the IL-6 receptor including activation of STAT1 and STAT3 [151]. STAT5, which is activated in T cells after IL-2 stimulation, is activated neither by the IL-10 receptor or following IL-2 receptor activation of B-CLLs. Taken together, differential activation of STAT1 and STAT3 proteins seems to be a critical signal event in cell survival for B-CLL cells. Nevertheless, there is no constitutive tyrosine phosphorylation on any STAT in CLL cells. Interestingly, Frank and colleagues [152] have reported that, apart from normal B lymphocytes, all CLL patients tested had STAT 1 and STAT3 proteins which were constitutively phosphorylated on serine residues 727. Thus the serine phosphorylation of these STATs seems to be a specific event for CLL. It is thought that serine phosphorylation which is not sufficient to activate STATs can augment gene transcription induced by tyrosine-phosphorylated STATs. In this model, signaling events initiated by cytokines such as IL-10 and IFNs are always maximal, and that the STAT-mediated responses cannot be further modulated. Future investigations will focus on the identification of the CLL STAT serine kinase(s) which seem to be a crucial trigger in the pathogenesis of CLL.

Therapeutic implications

Successful treatment of CLL patients with chemotherapy may require therapeutic agents that are able to induce apoptosis in the (predominantly) resting malignant B cells. This may explain why such drugs as topoisomerase II in-

hibitor and purine analogues which are potent inducers of apoptosis in tumor cells are among the most effective cytostatic agents in CLL. For example, the preponderant mechanism of action of the purine analog fludarabine seems to be its capacity to induce apoptosis in CLL cells. This effect is enhanced by combination with other drugs (cyclophosphamide, mitoxantrone, topoisomerase II inhibitors) which also induce apoptosis or synergize by various mechanisms [153].

A different approach is the attempt to drive the CLL cell out of its quiescent state. Loxoribine (a 7,8-disubstituted guanine ribonucleoside immunostimulant) is a new substance inducing CLL cells to enter and traverse the cell cycle. Furthermore, prolonged exposure of CLL cells to loxoribine frequently leads to progression of the responsive cells to apoptosis. This ability to induce cell cycle entry of otherwise G_0/G_1 arrested cells could render them more sensitive to conventional cytotoxic therapy [154, 155].

Identification of specific defects in CLL cell signaling such as the recent finding of constitutive phosphorylation of STATs could open new therapeutic targets such as specific inhibitors of the putative STAT serine kinases, ultimately leading to new treatment options [152].

Clearly, more research is needed to define new mechanisms of survival and growth of CLL cells in order to develop more specific treatment modalities which target the molecular events of leukemogenesis.

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Table 2. Effects of simvastatin on trabecular bone volume and bone formation rates. Simvastatin was given in doses of 5 to 50 mg/kg/day by oral gavage for 35 days to (i) 3-month-old virgin female rats (experiment 1), (ii) 3-month-old virgin female rats that had been ovariectomized within 7 days after the start of treatment (experiment 2), and (iii) 3-month-old virgin female rats that had been ovariectomized 2 months before treatment (experiment 3). In each experiment, the rats were weight matched and divided into treatment groups of 10. The rats were lightly anesthetized with isofluorane before ovariectomy. Animals were pair fed throughout the experimental period and body weights were determined weekly. Values in parentheses are percent change from vehicle-treated controls. BV/TV, bone volume/tissue volume; Ocl, osteoclasts; BFR, bone formation rate; OVX/veh, ovariectomized rats treated with vehicle; hPTH, human PTH; ND, not determined.

Treatment	Trabecular bone volume (% BV/TV)	BFR ($\mu m^3/\mu m^2$/day)	No. of Ocl/mm² of bone surface
	Experiment 1		
Control	13.4 ± 1.4		13.7 ± 1.2
Simvastatin (10 mg/kg/day)	18.6 ± 1.4* (+39)	ND	11.6 ± 1.4 (−15)
hFGF-1 (100 μg/kg/day)	21.4 ± 1.7* (+60)	ND	7.5 ± 1.3* (−45)
	Experiment 2		
OVX/veh	6.9 ± .87	0.6 ± 0.1	8 ± 0.2
Simvastatin (1 mg/kg/day)	8.6 ± .41 (+25)	ND	ND
Simvastatin (10 mg/kg/day)	13.4 ± 2* (+94)	1.2 ± .11 (100*)	7 ± 0.3 (−12.5)
	Experiment 3		
OVX/veh	4.6 ± 0.58	0.151 ± 0.01	1.2 ± 0.1
Simvastatin (5 mg/kg/day)	9 ± 0.8* (+96)	0.196 ± .021* (30)	0.9 ± 0.1 (−25)
Simvastatin (10 mg/kg/day)	8.6 ± 0.9* (+87)	0.229 ± .034* (52)	0.78 ± .06* (−33)
hPTH (80 μg/kg/day)	20 ± 1.9* (+348)	0.228 ± .025* (51)	0.84 ± 0.15 (−30)

*Significantly greater than control ($P < 0.01$).

with this process may lead to osteoclast apoptosis and cessation of bone resorption (*18, 20*). We cannot exclude the possibility that the statins both inhibit bone resorption and promote bone growth, and we did observe a concomitant decrease in osteoclast numbers (Table 2). However, this effect appeared minor in comparison to the effect on new bone formation and osteoblast maturation.

The statins used in our studies and currently on the market are not ideal for use as systemic bone-activation agents. These statins were selected for their capacity to lower serum cholesterol, which requires targeting to HMG Co-A reductase in hepatic cells. Thus, the concentration of statin in other tissues is much lower than in the liver. The most efficacious statins would be those that distribute themselves to the bone or bone marrow. A preliminary retrospective analysis of older women taking lipid-lowering agents suggests that statin use is accompanied by greater hip bone mineral density and lower risk of hip fractures (relative risk = 0.30) (*21*); however, the sample size (598 statin users) was too small to yield definitive information.

The most powerful anabolic agents for bone are the peptide growth factors intrinsic to the tissue. For example, systemically administered FGF-1 restores trabecular microarchitecture and increases bone volume (*15*). However, all of the peptide growth factors have disadvantages—they can be mitogenic to other bone cells and nonselective in their effects. In addition, the FGFs cause hypotension, which limits their potential use in elderly patients (*22*).

Our results suggest that statins, which are orally bioavailable and have been safely administered to patients for more than a decade, may merit further investigation as potential anabolic agents for bone. When the doses are extrapolated from humans to rats with respect to lipid lowering, the statins' effects on bone occur at doses similar to the lipid-lowering doses used in humans.

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Requirement for B Cell Linker Protein (BLNK) in B Cell Development

Rajita Pappu,[1,2] Alec M. Cheng,[1,2,3] Bin Li,[1,2,4] Qian Gong,[1,2,4] Chris Chiu,[1,2] Nancy Griffin,[1,2,4] Mike White,[1,3] Barry P. Sleckman,[1,3] Andrew C. Chan[1,2,3,4]*

Linker proteins function as molecular scaffolds to localize enzymes with substrates. In B cells, B cell linker protein (BLNK) links the B cell receptor (BCR)–activated Syk kinase to the phosphoinositide and mitogen-activated kinase pathways. To examine the in vivo role of BLNK, mice deficient in *BLNK* were generated. B cell development in *BLNK*$^{-/-}$ mice was blocked at the transition from B220^{+}CD43^{+} progenitor B to B220^{+}CD43^{-} precursor B cells. Only a small percentage of immunoglobulin M^{-*} (IgM^{*-}), but not mature IgM^{lo}IgD^{hi}, B cells were detected in the periphery. Hence, BLNK is an essential component of BCR signaling pathways and is required to promote B cell development.

Engagement of the BCR activates distinct families of cytoplasmic protein tyrosine kinases (PTKs) to phosphorylate enzymes that are required for the generation of second messengers (*1*). In turn, the coordinate generation of second messengers is important for normal B cell function because disruption of selected signaling pathways is associated with B cell anergy (*2*). Linker or adapter molecules play integral roles in linking the BCR-activated PTKs with these enzymes. One such linker molecule, BLNK (also known as SLP-65, BASH, and BCA), is phos-

[1]Center for Immunology; [2]Division of Rheumatology, Department of Medicine; [3]Department of Pathology; [4]Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110, USA.

*To whom correspondence should be addressed. E-mail: achan@im.wustl.edu

phorylated by Syk after BCR activation and interacts with enzymes, including phospholipase C-γ, Bruton's tyrosine kinase, and Vav (a guanine nucleotide exchanger for the Rho-GTPases), as well as the Grb2 and Nck linker proteins (*3–5*). An essential role for BLNK in BCR activation was demonstrated in a chicken *BLNK*$^{-/-}$ DT40 cell line that cannot increase the intracellular calcium concentration ($[Ca^{2+}]_i$) or efficiently activate the Erk-, JNK-, and p38-mediated signaling pathways (*6*).

To better define the expression pattern of BLNK, we developed an intracellular fluorescence-activated cell sorting (FACS) staining assay for BLNK. Consistent with earlier reports (*3, 5*), BLNK expression was detected in peripheral B, but not T, lymphocytes (*7*) (Fig. 1A). Analysis of bone marrow–derived cells showed the highest BLNK expression in early development, with progressively lower expression during B cell maturation (*7, 8*) (Fig. 1B). Hence, BLNK is expressed throughout B cell ontogeny and suggests a potential role for BLNK in B cell development, maturation, or function.

To investigate the in vivo role of BLNK, we undertook a gene-targeting approach to generate and analyze *BLNK*$^{-/-}$ mutant mice. Because BLNK is a substrate of Syk and *syk*$^{-/-}$ mice hemorrhage extensively in utero and die during the perinatal period (*3, 9*), we were concerned that *BLNK*$^{-/-}$ mice might suffer a similar fate. In addition, gene targeting of the BLNK homolog, *SLP-76*, results in mice that die from hemorrhage caused by a defect in collagen-induced platelet aggregation (*10*). To circumvent the embryonic lethality that may be encountered in germ line knockout mice, we also used the *RAG2*$^{-/-}$ blastocyst complementation system to assay for BLNK function in lymphocytes (*11*). The data presented here represent analyses from both approaches.

Disruption of *BLNK* was accomplished by a targeted mutation of exon 1, which encodes amino acids 1 through 60, including the initiation codon (*12*) (Fig. 2A). For *RAG2*$^{-/-}$ blastocyst complementation, the *BLNK*$^{+}$ allele in the *BLNK*$^{+/-}$ embryonic stem (ES) clone was further targeted with a puromycin selection cassette to generate *BLNK*$^{-/-}$ ES cells (*13*) (Fig. 2B). To generate germ line mutant mice, *BLNK*$^{+/-}$ ES cells were injected into B6 blastocysts to yield chimerae that were then crossed with wild-type B6 mice to generate *BLNK*$^{+/-}$ germ line mutants. The genotypes of the mature offspring from such crosses occurred at the expected Mendelian ratios and the *BLNK*$^{-/-}$ mutation did not incur any embryonic or perinatal lethality (*14*). Furthermore, mature *BLNK*$^{-/-}$ mice were healthy under specific pathogen-free conditions and did not display any evidence of gross hemorrhaging (*14*). To evaluate the developmental potential of *BLNK*$^{-/-}$ ES cells, we also injected these cells into *RAG2*$^{-/-}$ blastocysts to generate chimerae (*R2:BLNK*$^{-/-}$) for direct analysis (*15*).

To assess the effect of the mutation on BLNK expression, we used the Ly 9.1 surface marker to distinguish between bone marrow cells derived from the *RAG 2*$^{-/-}$ blastocyst (Ly 9.1^{-}) and the *BLNK*$^{-/-}$ ES cells (Ly 9.1^{+}) (*16*). Whereas the B220^{+}Ly 9.1^{+} cells from wild-type 129 mice and B220^{+}Ly9.1^{+} cells from *RAG2*$^{-/-}$ mice expressed BLNK (Fig. 2D, left two bottom panels), no BLNK was detected in the B220^{+}Ly9.1^{+} cells isolated from the *R2:BLNK*$^{-/-}$ chimerae, as assessed by intracellular staining (Fig. 2D, bottom right panel). Similar to the results from the *R2:BLNK*$^{-/-}$ chimerae, no BLNK protein was detected in cell lysates of total bone marrow from germline *BLNK*$^{-/-}$ mice (*17*) (Fig. 2E).

The effects of BLNK deficiency on lymphocyte development in vivo was examined by analyzing cells isolated from primary and secondary lymphoid organs. Consistent with the absence of BLNK expression in T cells, T cell number, development, and function were normal in both *BLNK*$^{-/-}$ germ line and *R2:BLNK*$^{-/-}$ chimeric mice (*14, 18–20*). In contrast, an ~65% reduction in splenocyte number was found in *BLNK*$^{-/-}$ mice as compared to *BLNK*$^{+/+}$ or $^{+/-}$ mice (*18*). Because the development and function of peripheral T cells were normal (*14, 19*), we further investigated the nature of this defect by analyzing the B cell compartment in primary and secondary lymphoid organs. Although the numbers of cells recovered from the bone marrow of *BLNK*$^{+/+}$ and *BLNK*$^{-/-}$ mice were similar (*18*), bone marrow cells from *BLNK*$^{+/-}$ germ line and *R2:BLNK*$^{-/-}$ chimeric mice displayed a profound block in B cell devel-

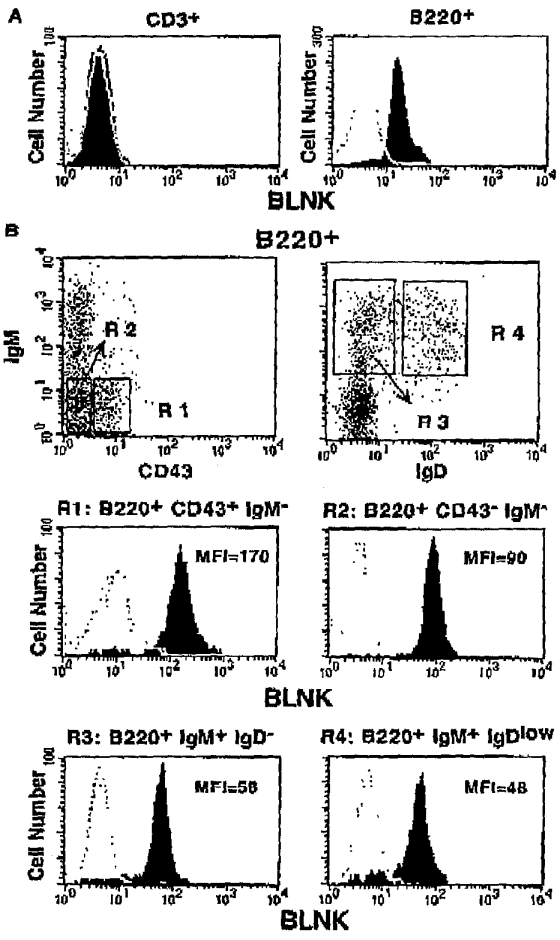


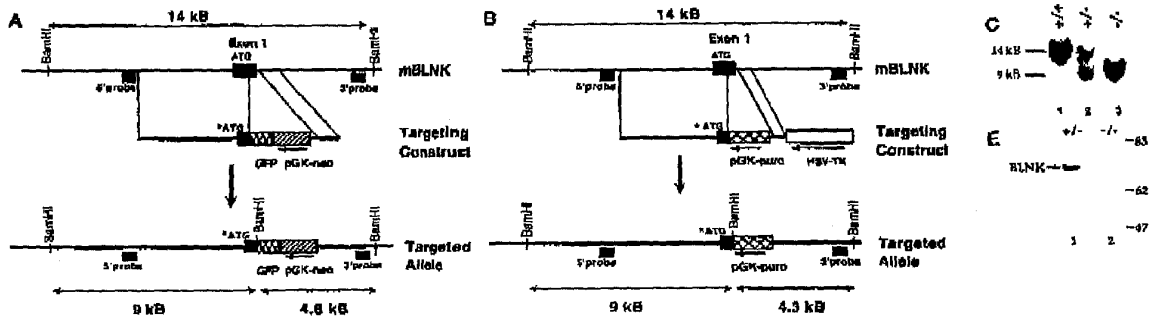
Fig. 1. Expression of BLNK in lymphocyte development. (A) BLNK is expressed in murine B, but not T, cells. CD3^{+} (left panel) or B220^{+} (right panel) splenocytes isolated from C57BL/6 mice were analyzed by intracellular staining with an antiserum to BLNK (shadowed areas) or preimmune serum (solid line) (*7*). (B) BLNK expression during murine B cell development. Bone marrow–derived cells isolated from C57BL/6 mice were analyzed with four-color FACS analysis (*8*). Cells stained for B220, IgM, and CD43 (left panel) or for B220, IgM, and IgD (right panel) were analyzed as described above. Each developmental subset—B220^{+} CD43^{+}IgM^{-} (pro-B cells; R1), B220^{+}CD43^{-}IgM^{-} (pre-B cells; R2), B220^{+} IgM^{+} IgD^{-} (immature B cells; R3), and B220^{+} IgM^{+}IgD^{hi} (mature B cells; R4)—was analyzed for BLNK expression (*7*).

opment. *BLNK*$^{-/-}$ mice accumulated B220^{+}CD43^{+} progenitor B cells (pro-B cells) (*21*) (Fig. 3A). Consistent with the presence of pro-B cells, the levels of V_H to DJ_H recombination were comparable in *BLNK*$^{+/+}$ and *BLNK*$^{+/-}$ bone marrow–derived cells (*14*). *BLNK*$^{-/-}$ mice had CD43^{+} pro-B cells but failed to develop B220^{hi}CD43^{-} B cells, although a small percentage of B220^{+}CD43^{-} B cells was present (10.0 ± 8.7% for *BLNK*$^{-/-}$ versus 44.4 ± 14% for *BLNK*$^{+/-}$ or $^{+/+}$, $P < 0.001$, $n = 11$) (*20*) (Fig. 3A). Because the transition from the CD43^{+} to CD43^{-} stage is normally associated with a decrease in cell size, as measured by the forward scatter value (*8*), the B220^{-}CD43^{-} B cells isolated from *BLNK*$^{-/-}$ mice remained large, in contrast to the smaller B220^{+}CD43^{-} B cells from *BLNK*$^{+/-}$ mice (*14*). In addition, the *BLNK*$^{-/-}$ bone marrow (B lineage) cells failed to progress efficiently from the immature B220^{lo}IgM^{lo} (immunoglobulin M, IgM) stage to transitional B220^{lo}IgM^{hi} or mature B220^{hi}IgM^{+} stages (1.3% ± 0.9% in *BLNK*$^{-/-}$ mice for the latter two stages versus 13.1 ± 5 in *BLNK*$^{+/+}$ or $^{+/-}$ mice, $P < 0.001$, $n = 10$) (*20*) (Fig. 3A). The small percentage of IgM^{lo} bone marrow B cells that develop in *BLNK*$^{-/-}$ mice express a mature surface BCR because many are also Igκ^{+} (*14*).

Analysis of splenocytes revealed a substantial decrease in the numbers of IgM^{+} peripheral B cells (2.4 ± 2.6% for *BLNK*$^{-/-}$ versus 30.7 ± 6.2% for *BLNK*$^{+/+}$ or $^{+/-}$, $P < 0.001$, $n = 10$) (*20*) (Fig. 3B). As in the bone marrow, the few *BLNK*$^{-/-}$ IgM^{+} B cells found in the spleen were also larger in size than IgM^{hi} *BLNK*$^{+/-}$ B cells (*14*). Concomitant with the profound decrease in peripheral B cells in the spleen, IgM^{+} B cells were also reduced in the lymph node (Fig. 3C). Hence, the absence of BLNK results in a developmental block that leads to reduced numbers of IgM^{+} cells in the periphery. Older *BLNK*$^{-/-}$ mice (8 to 13 weeks old) showed increased numbers of B220^{+}IgM^{+} B cells [(1.4 ± 1.2) × 10^{6} B220^{+}IgM^{+} cells, $n = 8$] as compared to younger *BLNK*$^{-/-}$ mice [3 to 6 weeks old; (0.58 ± 0.31) × 10^{6}, $P < 0.001$, $n = 9$]. In spite of this accumulation, these older *BLNK*$^{-/-}$ mice still have more than 10 times fewer B220^{+}IgM^{+} B cells than their age-matched *BLNK*$^{+/+}$ or $^{+/-}$ counterparts [(19 ± 6.7) × 10^{6} B220^{+}IgM^{+} cells in older *BLNK*$^{+/+}$ or $^{+/-}$ mice, $n = 6$, versus (1.4 ± 1.2) × 10^{6} B220^{+}IgM^{+} cells in older *BLNK*$^{-/-}$ mice, $P < 0.001$, $n = 8$] (*20*).

Analysis for mature B cells revealed a marked reduction of B220^{hi}IgM^{+} cells (<1%) in the bone marrow of young and old *BLNK*$^{-/-}$ mice (Fig. 3A) (*14*). Mature IgM^{lo}IgD^{hi} cells were similarly reduced (<1%) in the periphery of young and old *BLNK*$^{-/-}$ mice (Fig. 4, A and B). Staining with CD21 revealed the presence of CD21^{+}IgM^{hi} T2 transitional B cells and a reduction of CD21^{+}IgM^{lo} mature B cells (<1%) in *BLNK*$^{-/-}$ mice (*22*) (Fig. 4B). Consistent with the decrease in mature B cells, serum Ig in older *BLNK*$^{-/-}$ mice was significantly reduced as compared to the amount in wild-type mice (*23*) (Fig. 4C).

The *BLNK*$^{-/-}$ B cells that accumulated in the periphery of older mice further revealed a maturation defect in these cells. In contrast to *BLNK*$^{+/-}$ mice, in which transitional B220^{+}IgM^{++}IgD^{+} B cells develop into mature B220^{hi}IgM^{lo}IgD^{hi} B cells, and in contrast to the B cells that accumulate in the λ5$^{-/-}$ mice (*24*), *BLNK*$^{-/-}$ splenic B cells are larger in size and express higher mem-



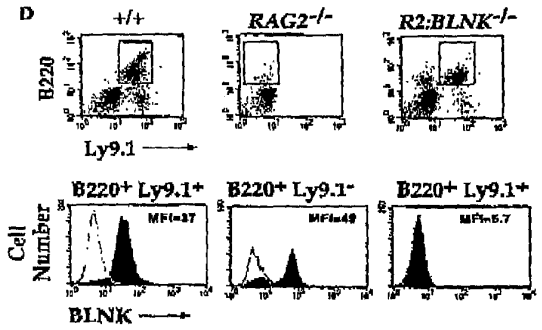


Fig. 2. Generation of *BLNK*$^{-/-}$ mice. **(A)** Targeting of *BLNK*. The genomic structure surrounding exon 1 of *BLNK* (top), the targeting construct (middle), and the targeted allele (bottom) are depicted (*12*). Exon 1 includes amino acids 1 through 60 of the BLNK coding region. The correctly integrated construct converts the 14-kb wild type into 9- and 4.8-kb fragments when detected with the 5′ and 3′ probes, respectively. A GFP cDNA was also inserted into the targeting construct. However, GFP fluorescence was not detected in *BLNK*$^{+/-}$ splenocytes or bone marrow–derived cells, which was likely caused by transcriptional silencing of GFP by the PGK-neo cassette (*12*). **(B)** Targeting of the second *BLNK* allele. The genomic structure surrounding exon 1 of *BLNK* (top), the targeting construct (middle), and the targeted allele (bottom) are depicted (*13*). The correctly integrated construct converts the remaining 14-kb wild type into 9- and 4.3-kb fragments when detected with the 5′ and 3′ probes, respectively. **(C)** Southern (DNA) blot analysis of *BLNK*$^{+/+}$, *BLNK*$^{+/-}$, and *BLNK*$^{-/-}$ mice. Bam HI–digested tail DNA was separated by electrophoresis and hybridized with the 5′ probe to detect the wild-type and mutant fragments (*13*). Blotting with the 3′ probe also revealed the predicted mutant 4.8-kb fragment in *BLNK*$^{+/-}$ and *BLNK*$^{-/-}$ mice (*14*). **(D)** Absence of BLNK protein in *RAG2*$^{-/-}$ chimeric mice. B220^{+} bone marrow cells from 129 wild-type (Ly9.1^{-}; left panels), *RAG2*$^{-/-}$ (Ly9.1^{-}; middle panels), or *R2:BLNK*$^{-/-}$ chimeric (Ly9.1^{-}; right panels) mice were analyzed by intracellular staining for BLNK as described in Fig. 1A (*7*). **(E)** Absence of BLNK protein in *BLNK*$^{-/-}$ bone marrow–derived cells. Bone marrow–derived cells from germ line *BLNK*$^{+/-}$ (lane 1) and *BLNK*$^{-/-}$ (lane 2) littermates were immunoblotted with an antiserum to BLNK (*17*). Equal loading of cell lysates was confirmed by immunoblotting with an antiserum to actin (Sigma) (*14*).

brane IgM (Fig. 4B) (*14*). These IgM^{hi} cells may represent B cells that have matured through the pro- to precursor B cell (pre-B cell) transition but are blocked in IgM signaling and, therefore accumulate as large IgM^{hi} cells. Alternatively, these cells may result from a selection bias in which B cells can bypass the absence of BLNK by increasing membrane Ig expression and decreasing the signaling threshold. As $CD45^{-/-}$ immature B cells expressing a transgenic BCR can be rescued from death by chronic exposure to antigen (*25*), heightened BCR signaling may bypass the requirement for CD45. Similarly, BLNK deficiency may abolish development and result in the death of most B cells, except those that express very large amounts of IgM, which partially compensates for the signaling defect incurred by BLNK deficiency. The IgM^{++} $BLNK^{-/-}$ B cells could increase free cytoplasmic calcium after BCR cross-linking,

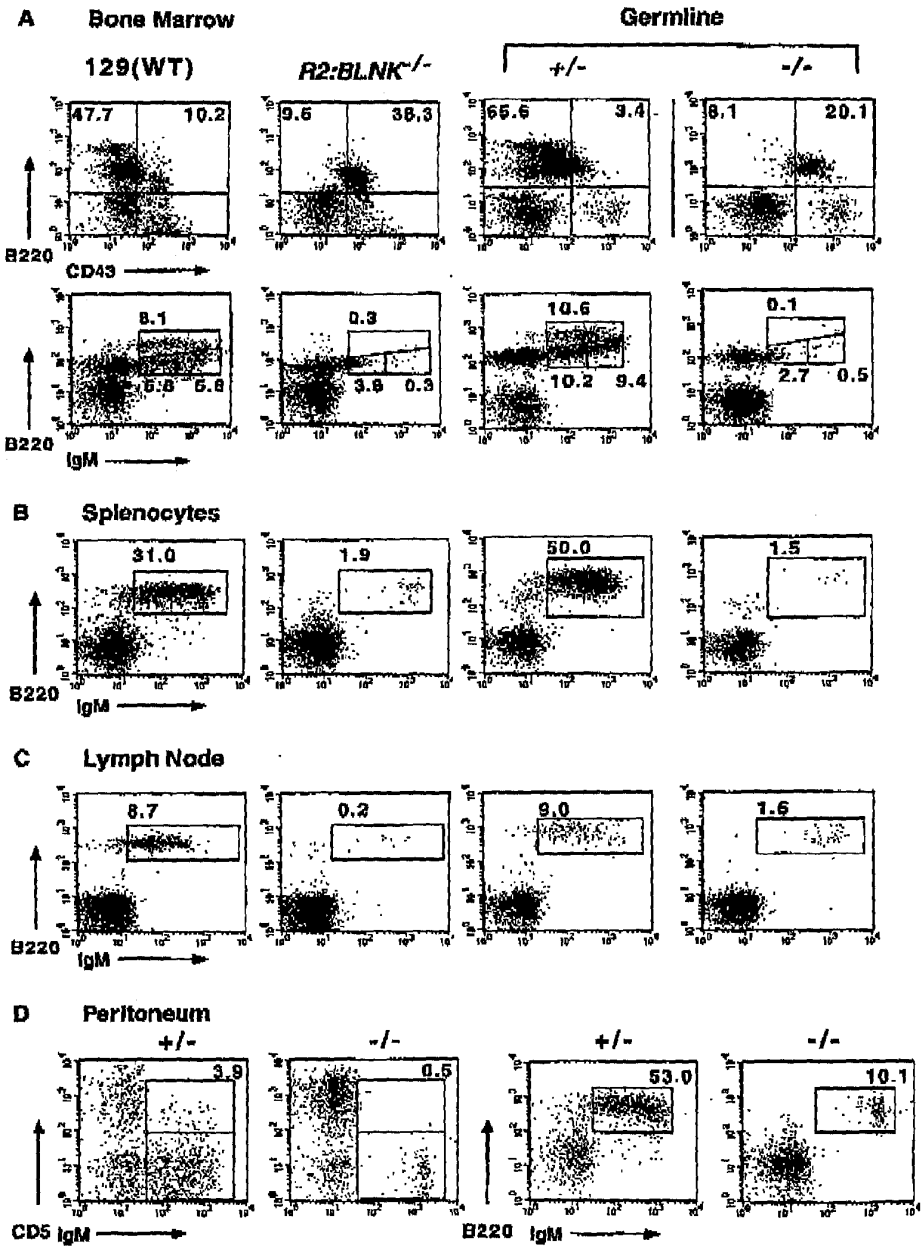


Fig. 3. B cell development in $BLNK^{-/-}$ mice. Cells isolated from bone marrow (A), spleen (B), and lymph nodes (C) of 3- to 5-week-old animals were stained with the antibodies indicated in each figure and analyzed by FACS analysis (*27*). Data from both *R2:BLNK*$^{-/-}$ chimeric [left two panels for (A) through (C)] and germ line [right two panels for (A) through (C)] mice are shown. In the *RAG2*$^{-/-}$ blastocyst complementation assay, 129 wild-type and *RAG2*$^{-/-}$ age-matched mice were analyzed in parallel as controls (*14*). No differences were detected between $BLNK^{+/+}$ and $BLNK^{+/-}$ mice (*14*). The percentages of gated cells are indicated. These analyses were representative of a minimum of five pairs each of *RAG2*$^{-/-}$ chimeric and germ line animals. Experiments from both approaches produced similar results. (D) Peritoneal cells isolated from 6- to 13-week-old mice were stained with the antibodies indicated and analyzed by FACS analysis (*27*). Cell recoveries were comparable in yield from $BLNK^{+/-}$ and $BLNK^{-/-}$ mice [4.5 × 10^6 for $BLNK^{+/-}$ and (3.2 ± 0.6) × 10^6 for $BLNK^{-/-}$ mice, n = 5].

albeit less efficiently than wild-type cells, despite expressing 10- to 50-fold more membrane IgM than $BLNK^{+/+}$ B cells (*26*) (Fig. 4D). In addition, $BLNK^{-/-}$ B cells also up-regulate CD69 and CD86 cell surface activation markers after BCR cross-linking (*27*) (Fig. 4E). Hence, these large IgM^{++} $BLNK^{-/-}$ B cells are capable of some BCR-mediated signaling functions.

We also analyzed the development of a distinct subset of B cells known as B-1a cells. These cells are distinguished from conventional B-2 cells by their expression of CD5 and their capacity for self-renewal (*28*). Whereas $BLNK^{+/+}$ and $BLNK^{+/-}$ mice had comparable numbers of peritoneal cells, $BLNK^{-/-}$ mice had a substantial decrease in the $CD5^{+}IgM^{+}$ B-1a B cell population (<1%) in the peritoneum in young and old mice (Fig. 3D) (*14*). In addition, $CD5^{-}CD11b^{+}IgM^{+}$ B-1b B cells were also absent (<0.5%) in the peritoneum and the spleen of $BLNK^{-/-}$ mice (*14, 29*). Hence, BLNK is also required for development of B-1 cells.

Because signals from both the pre-B and IgM BCRs are required for normal B cell development (*1, 30*), these studies showed the critical role of BLNK in the development of IgM^{+} cells. Similar to $syk^{-/-}$ mice (*9*), the absence of BLNK also compromises pre-BCR function to affect the development of $B220^{+}CD43^{-}$ B cells that, in turn, limit their differentiation into $B220^{hi}IgM^{+}$ B cells in the bone marrow. As a result, few B cells are present in the periphery. However, whereas the IgM^{+} B cells that develop in $syk^{-/-}$ mice express little membrane IgM (*9*), the B cells that accumulate in $BLNK^{-/-}$ mice express large amounts of membrane IgM (Fig. 4B). This difference suggests that additional substrates of Syk might exist to partially transduce pre-BCR signals in the absence of BLNK. In accordance with this, the IgM^{++} B cells that accumulate in the periphery of older $BLNK^{-/-}$ mice can generate second messengers after BCR activation. Additional studies aimed at comparing $syk^{-/-}$ and $BLNK^{-/-}$ mice will be required to assess this possibility.

Finally, the developmental block at the pro- to pre-B cell transition observed in a BLNK-deficient patient is similar, though not identical, to the phenotype observed in $BLNK^{-/-}$ mice (*31*). Although IgM^{hi} B cells accumulate in the periphery of $BLNK^{-/-}$ mice, no peripheral B cells were detected in this adult patient. Similar discordance in phenotypes has been observed in immunodeficiencies involving Btk and λ5 in which the human phenotype appears to be more severe than the murine phenotype (*32*). These differences may reflect a greater dependence on pre-BCR function in human B cell development, a species-specific difference in the regulation of signaling molecules that dictate activation thresholds, or both. Such species-specific differences have been observed in T cell development in which Syk is more highly expressed in developing human $CD4^{+}$ T cells than in murine $CD4^{+}$ T cells and may provide a mechanism to explain the phenotypic differences observed between ZAP-70–deficient mice and humans (*33*). Additional investigation is required to determine whether species-specif-

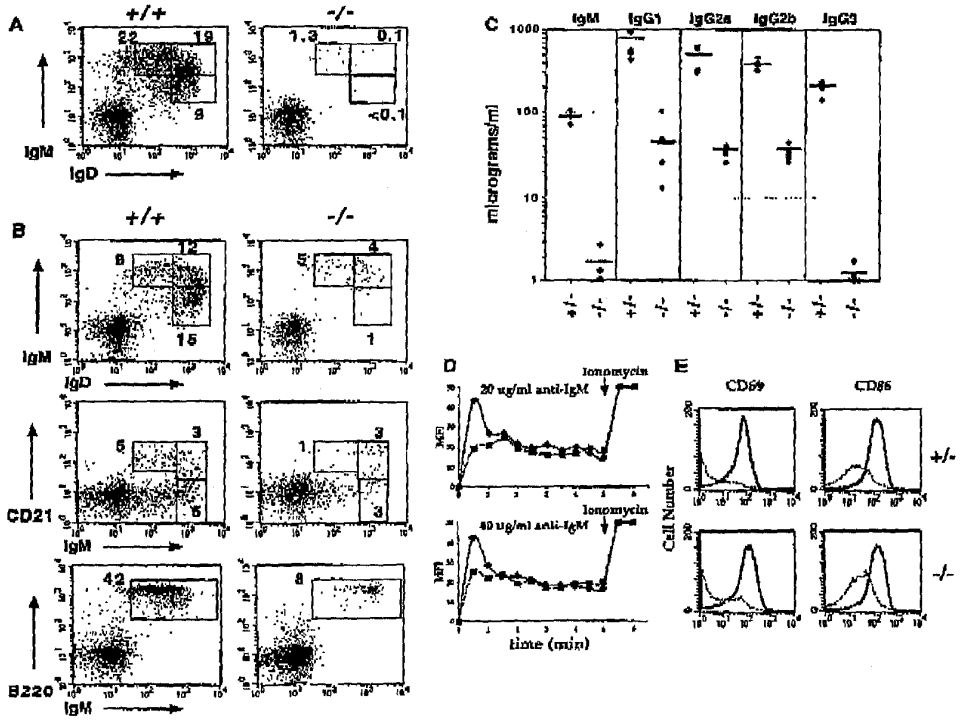


Fig. 4. Decreased maturation of B cell development and function in $BLNK^{-/-}$ mice. (**A**) Splenocytes isolated from 3- to 5-week-old $BLNK^{+/+}$ or $BLNK^{-/-}$ mice were analyzed by FACS staining for IgM and IgD (*27*). (**B**) Splenocytes from older $BLNK^{+/+}$ or $BLNK^{-/-}$ mice (8 to 13 weeks old) were stained with the antibodies indicated in each figure and analyzed by FACS analysis (*27*). The percentages of gated cells are indicated. $BLNK^{-/-}$ spleens were reduced in cell number by ~70% as compared to $BLNK^{+/+}$ spleens (*14, 18*) (see text for absolute numbers of cells recovered). (**C**) Serum immunoglobulin levels of 8- to 13-week-old wild-type, germ line $BLNK^{-/-}$ and *R2:*$BLNK^{-/-}$ mice were determined by ELISA (*23*). Each diamond represents the value derived from an individual mouse. The black bar denotes the mean of each group. (**D**) $B220^{+}$ B cells from older $BLNK^{+/+}$ (solid line) and $BLNK^{-/-}$ (dotted line) mice were analyzed by FACS analysis for increases in $[Ca^{2+}]_i$ after BCR cross-linking with antibody to IgM $F(ab')_2$ fragments (20 and 40 μg/ml) (*26*). Antibody to IgM $F(ab')_2$ was added at time 0 at the indicated concentrations. Ionomycin was added at 5 min to ensure proper loading of cells with Fluo-4. Diamond, $BLNK^{+/+}$ splenocytes; square, $BLNK^{-/-}$ splenocytes. (**E**) $B220^{+}$ B cells from older $BLNK^{+/+}$ (top) and $BLNK^{-/-}$ (bottom) mice were analyzed by FACS analysis for up-regulation of CD69 and CD86 expression after BCR cross-linking (*27*). The dotted line represents cells treated with media. The solid line represents cells treated with antibody to IgM $F(ab')_2$ (10 μg/ml).

ic differences in the regulation of BLNK or other regulators of B cell development may account for the differences observed between human and murine BLNK deficiencies. However, the present studies in a human and in mice demonstrate a central role for BLNK in relaying signals in the pre-BCR and BCR signaling pathways.

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7. Cells (4×10^6) were prepared with the CytoFix/CytoPerm Kit (PharMingen) according to manufacturer's recommendations. After permeabilization, cells were stained with 2 μg of an antiserum to BLNK (*3*) in buffer containing 50% fetal calf serum (FCS) at 4°C for 30 min. Cells were washed twice with 1 ml of wash buffer and stained with 1 μg of fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (heavy and light chains) (Jackson Research Labs) for 30 min at 4°C. Cells were washed twice with 1 ml of wash buffer, resuspended in 0.4 ml of cold phosphate-buffered saline, and prepared for FACS analysis.
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19. Supplementary Information is available at *Science* Online at www.sciencemag.org/feature/data/1045809.shl
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An Essential Role for BLNK in Human B Cell Development

Yoshiyuki Minegishi,[1] Jurg Rohrer,[1] Elaine Coustan-Smith,[2] Howard M. Lederman,[3] Rajita Pappu,[4] Dario Campana,[2,5] Andrew C. Chan,[4] Mary Ellen Conley[1,5]*

The signal transduction events that control the progenitor B cell (pro-B cell) to precursor B cell (pre-B cell) transition have not been well delineated. In evaluating patients with absent B cells, a male with a homozygous splice defect in the cytoplasmic adapter protein BLNK (B cell linker protein) was identified. Although this patient had normal numbers of pro-B cells, he had no pre-B cells or mature B cells, indicating that BLNK plays a critical role in orchestrating the pro-B cell to pre-B cell transition. The immune system and overall growth and development were otherwise normal in this patient, suggesting that BLNK function is highly specific.

Cross-linking of the B cell antigen receptor (BCR) results in rapid phosphorylation of the adapter protein BLNK [also called SLP-65 (Src homology 2 domain–containing leukocyte protein of 65 kD) and BASH (B cell adapter containing Src homology 2 domain)], a hematopoietic-specific cytoplasmic protein with homology to SLP-76 (*1*, *2*). Once BLNK is phosphorylated by Syk, it serves as a scaffold to assemble the downstream targets of antigen activation, including Grb2, Vav, Nck, and phospholipase C-γ (PLCγ). Hence, BLNK is positioned to coordinate a number of signaling pathways activated by the BCR. Studies in a

ic differences in the regulation of BLNK or other regulators of B cell development may account for the differences observed between human and murine BLNK deficiencies. However, the present studies in a human and in mice demonstrate a central role for BLNK in relaying signals in the pre-BCR and BCR signaling pathways.

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7. Cells (4×10^6) were prepared with the CytoFix/CytoPerm Kit (PharMingen) according to manufacturer's recommendations. After permeabilization, cells were stained with 2 μg of an antiserum to BLNK (3) in buffer containing 50% fetal calf serum (FCS) at 4°C for 30 min. Cells were washed twice with 1 ml of wash buffer and stained with 1 μg of fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (heavy and light chains) (Jackson Research Labs) for 30 min at 4°C. Cells were washed twice with 1 ml of wash buffer, resuspended in 0.4 ml of cold phosphate-buffered saline, and prepared for FACS analysis.
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Cross-linking of the B cell antigen receptor (BCR) results in rapid phosphorylation of the adapter protein BLNK [also called SLP-65 (Src homology 2 domain–containing leukocyte protein of 65 kD) and BASH (B cell adapter containing Src homology 2 domain)], a hematopoietic-specific cytoplasmic protein with homology to SLP-76 (*1*, *2*). Once BLNK is phosphorylated by Syk, it serves as a scaffold to assemble the downstream targets of antigen activation, including Grb2, Vav, Nck, and phospholipase C-γ (PLC-γ). Hence, BLNK is positioned to coordinate a number of signaling pathways activated by the BCR. Studies in a

BLNK-deficient DT40 chicken B cell line indicate that this adapter is required for the release of intracellular calcium and the activation of the extracellular signal-regulated protein kinase, c-Jun NH_2-terminal kinase, and p38 pathways in response to anti–immunoglobulin M (IgM) stimulation (*3*). If BLNK plays a nonredundant role in mammalian B cell development, mutations in BLNK might result in immunodeficiency.

About 85% of patients with early onset hypogammaglobulinemia and absent B cells are males with X-linked agammaglobulinemia (XLA) (*4*). These patients have mutations in the cytoplasmic tyrosine kinase Btk (*5*). Some of the remaining patients have defects in components of the pre-B cell receptor (pre-BCR) or BCR (*6, 7*); however, the nature of the defect in many patients remains unknown. To determine if mutations in BLNK could give rise to human immunodeficiency, we isolated and characterized a bacterial artificial chromosome clone containing the human genomic BLNK sequence. Fluorescence in situ hybridization demonstrated that BLNK is located on chromosome 10q23.22. The gene consists of 17 exons spread over ~65 kb of DNA. Primers were designed to amplify individual exons by polymerase chain reaction (PCR) for analysis by single-strand conformation polymorphism (SSCP) (*8*). Genomic DNA samples were analyzed from 25 patients with a Btk-deficient phenotype, in whom we had not identified mutations in Btk, μ heavy chain, Igα (mb-1), Igβ (B29), or the surrogate light chain. DNA from one patient, a 20-year-old male with early onset hypogammaglobulinemia and absent B cells, demonstrated a homozygous alteration in the first exon of BLNK and its flanking intronic sequence (Fig. 1). This portion of the gene was cloned and sequenced, and two noncontiguous base-pair substitutions were identified (*9*). The first alteration, a C to A substitution, occurred at the third base-pair position in codon 10, which encodes a proline. This base-pair substitution does not change the amino acid sequence of BLNK. The second alteration, an A to T substitution, was found at the +3 position of the splice donor site for intron 1, 20 base pairs downstream from the alteration in codon 10. SSCP analysis of DNA from 100 unrelated individuals did not reveal any fragments with a migration pattern identical to that seen in the patient (*10*).

[1]Department of Immunology, [2]Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA. [3]Division of Pediatric Allergy and Immunology, Department of Pediatrics, Johns Hopkins Hospital, Baltimore, MD 21287, USA. [4]Center for Immunology, Division of Rheumatology, Departments of Medicine and Pathology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110, USA. [5]Department of Pediatrics, University of Tennessee, College of Medicine, Memphis, TN 38105, USA.

*To whom correspondence should be addressed. E-mail: maryellen.conley@stjude.org

The A to T substitution at the +3 position of the splice donor site occurs at a highly conserved site in the splice consensus sequence; alterations at this site would be expected to result in faulty processing of the BLNK message (*11*). To evaluate this possibility, we derived cDNA from the patient's bone marrow and used reverse transcriptase–PCR (RT-PCR) to examine the abundance of BLNK transcripts (*12*). The results were compared with those obtained from bone marrow of healthy subjects or patients with mutations in Btk or μ heavy chain (Fig. 2). No BLNK transcripts could be amplified from the patient's bone marrow, although BLNK transcripts were easily identified in the bone marrow of the other patients with defects in early B cell development. Other genes expressed in pro-B cells, including Btk, terminal deoxynucleotidyl transferase (TdT), and λ5, were expressed in approximately equal amounts in all of the patients. These results indicate that the base-pair substitutions in BLNK resulted in a marked reduction or absence in BLNK transcripts and therefore in BLNK protein.

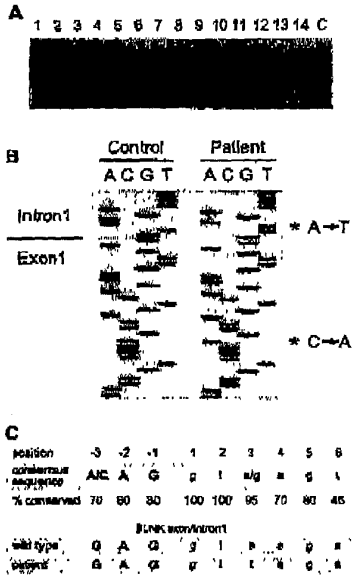

Fig. 1. Characterization of the BLNK mutation in an immunodeficient patient. (A) Genomic DNA samples from 14 patients with defects in B cell development and a control (lane C) were analyzed by SSCP for defects in the first exon of BLNK and its flanking sequences. DNA from the patient is shown in lane 2. (B) Sequence analysis at the exon-intron border demonstrated two base-pair substitutions, as indicated. (C) The consensus sequence for a mammalian 5′ splice donor site is shown with the wild-type and mutant BLNK exon 1/intron 1 sequence. The coding sequence is shown in capital letters; the intronic sequence is in lowercase letters.

The patient with BLNK deficiency demonstrated normal growth and development. At 8 months of age, he had the onset of recurrent otitis. After two episodes of pneumonia, he was evaluated for immunodeficiency at 16 months of age. At that time, he had no detectable serum IgG, IgM, or IgA, and he had <1% B cells in the peripheral circulation. He was started on gammaglobulin replacement, and between 2 and 20 years of age, he did well except for chronic otitis and sinusitis, hepatitis C acquired from intravenous gammaglobulin, and an episode of protein-losing enteropathy in adolescence. Immunologic studies performed when the BLNK-deficient patient was 20 years of age demonstrated serum concentrations of IgM and IgA of <7 mg/dl, normal numbers and percentages of CD4 and $CD8^+$ T cells and natural killer cells, and normal numbers of platelets and myeloid cells. The patient's mother and father, who were heterozygous for both base-pair substitutions in BLNK, were healthy and had normal concentrations of serum immunoglobulins and normal numbers of B cells (*13*). An older brother developed recurrent otitis at 6 months of age and died at 16 months of age of pseudomonas sepsis and neutropenia.

Immunofluorescence analysis of peripheral blood lymphocytes from the patient with BLNK deficiency and an age-matched patient with an amino acid substitution in the pleckstrin homology domain of Btk demonstrated that both patients had <0.01% $CD19^+$ cells in the blood (*14*). To determine the point in B cell differentiation at which the block in develop-

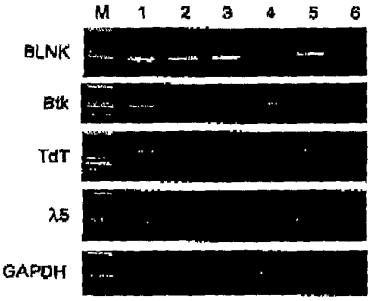

Fig. 2. RT-PCR analysis of B cell–specific transcripts in patients with defects in B cell development. RNA from the following sources was reverse transcribed: the bone marrow of a normal control (lane 1), a patient with an amino acid substitution in codon 113 of Btk (lane 2), a patient with a 4-bp deletion in the coding sequence of Btk (lane 3), the patient with mutations in BLNK (lane 4), and a patient with an amino acid substitution at an invariant cysteine in CH4 of μ heavy chain (lane 5). The cDNA was used as a template for RT-PCR with primers specific for the coding regions of BLNK, Btk, TdT, λ5, and the control transcript, GAPDH. Molecular weight markers are shown on the left (lane M), and a cDNA negative control is shown on the right (lane 6).

ment occurred, we examined bone marrow from both patients using markers that distinguish pro-B cells from pre-B cells and mature B cells. The percentage of $CD19^+$ B lineage cells was less in the patients in comparison to that of the control (0.3% in the BLNK-deficient patient and 1.0% in the Btk-deficient patient versus 15.7% in the control). There were no membrane immunoglobulin-positive (mIg^-) mature B cells in either patient (Fig. 3). In both patients, the block in B cell differentiation occurred at the pro-B cell to pre-B cell transition; >80% of the $CD19^+$ cells from these patients coexpressed the pro-B cell marker, CD34. In contrast, only 22.0% of the $CD19^+$ cells from the control were positive for CD34; the remaining cells from the control were either pre-B cells ($CD34^-$, $CD19^+$, and mIg^-) or B cells ($CD34^-$, $CD19^+$, and mIg^+).

To document that BLNK is expressed in pro-B cells, we indirectly stained permeabilized bone marrow cells from the Btk- and BLNK-deficient patients with a monoclonal antibody to BLNK (*14*). All of the $CD19^+$ pro-B cells from the Btk-deficient patient were positive for BLNK (Fig. 4). By contrast, there was little or no staining for BLNK in the bone marrow of the patient with mutations in BLNK. Because BLNK is expressed in pro-B cells, the possibility that BLNK is required before the expression of the pre-BCR was examined. In previous studies, we have shown that patients with defects in the constant region of the μ heavy chain or the Igα signal transduction component of the BCR have small amounts of transcripts for rearranged μ heavy chain genes in the bone marrow as detected by RT-PCR (*7*). Rearrangement of the μ heavy chain occurs immediately before the pro-B cell to pre-B cell transition. A primer that hybridizes to a conserved sequence within framework region 3 of variable-region genes and a primer within the CH1 domain of μ heavy chain were used to examine cDNA from a control and patients with defects in B cell development (*7*). A small number of rearranged μ heavy chain transcripts could be detected in the bone marrow of the patient with mutations in BLNK as well as in patients that were Btk and μ heavy chain deficient. Thus, BLNK does not play a role in B cell development before the expression of the pre-BCR. This corresponds with earlier studies showing that phosphorylation of BLNK is dependent on cell surface expression of a BCR (*15*).

Cell surface expression of the pre-BCR results in a strong survival signal associated with the cessation of μ heavy chain gene rearrangements, changes in cell surface phenotype, and marked expansion of the pre-B cell population (*16*). The absence of pre-B cells or B cells in the patient with mutations in BLNK demonstrates that BLNK plays a critical role in orchestrating these signals. Like defects in Btk and λ5 (*3, 6, 17*), mutations in BLNK appear to have more severe consequences in the human as compared to the mouse (*18*). This suggests that the requirements for signaling through the pre-BCR and BCR may be more stringent in the human than in the mouse. There may be a reciprocal reliance on signaling through other pathways in murine B cell development. For example, the consequences of defective signaling through interleukin-7 are more severe in the mouse as compared to the human (*19*).

In T cells, the functions performed by BLNK appear to be split between LAT (linker for activation of T cells), which binds to phosphatidylinositol 3-kinase, Grb-2, and PLCγ (*20*), and SLP-76, which binds GrpL, Nck, Vav, and FYB (FYN binding protein) (*21*). Mice lacking LAT (*22*) or SLP-76 (*23, 24*) have a block in T cell development at the pro-T to pre-T cell stage of development. In the newborn period, SLP-76–deficient mice also develop a hemorrhagic diathesis, which is related to the requirements for SLP-76 in collagen-mediated platelet activation (*24, 25*). These studies, when coupled with our findings showing that BLNK is required for normal B cell development in the human and the mouse, indicate that adapter proteins play a critical role in highly specific signaling pathways, and they suggest that defects in adapter proteins like LAT or SLP-76 may result in human immunodeficiency.

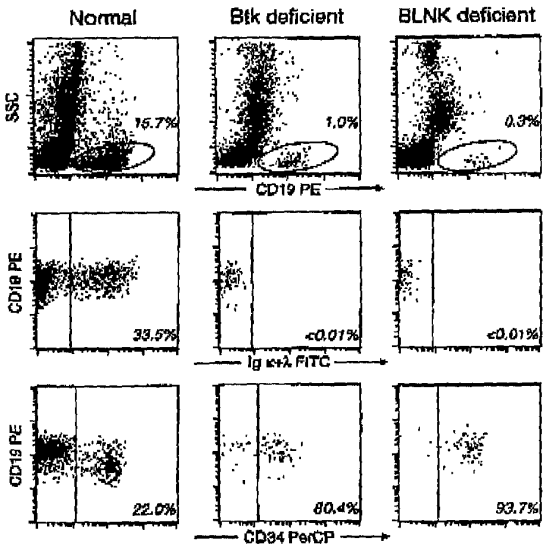

Fig. 3. Immunofluorescence analysis of B lineage cells. Bone marrow mononuclear cells from a normal individual (left column), from a patient with Btk-deficient XLA (middle column), and from the BLNK-deficient patient (right column) were labeled with antibody to CD19 PE, antibody to CD34 PerCP, and antibody to Ig κ and λ light chains FITC. Flow cytometric dot plots in the top row illustrate CD19 staining versus side scatter (SSC); both patients had reduced proportions of $CD19^+$ cells. Gated $CD19^+$ lymphoid cells were then analyzed for expression of mIg light chains (middle row) and CD34 (bottom row). Percentages of mIg^+ and $CD34^+$ among $CD19^+$ cells are indicated.

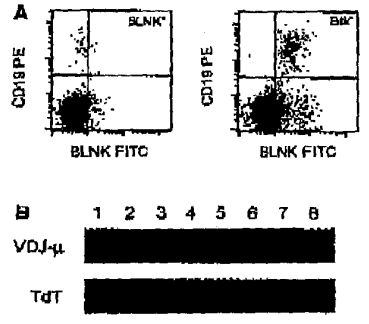

Fig. 4. (A) BLNK protein expression in pro-B cells. Bone marrow mononuclear cells from age-matched patients with BLNK (left) or Btk (right) deficiency were labeled with antibody to CD19 (IgM), permeabilized, and labeled with monoclonal antibody to BLNK (IgG2a). Goat antibody to mouse IgM PE and IgG FITC was then added. Dot plots illustrate immunofluorescence staining of lymphoid cells. Quadrants were set at the upper limits of the isotype-matched nonreactive antibody fluorescence. The $BLNK^+$ $CD19^-$ cells seen in the Btk-deficient patient were $CD34^-$ and were similar to monocytes in forward and side light scatter. (B) Semiquantitative RT-PCR analysis to evaluate the amount of VDJ-rearranged μ heavy-chain transcripts. Bone marrow cDNA from an age-matched control (lane 1), a patient with XLA (lane 2), the patient with BLNK deficiency (lane 3), a patient with μ heavy chain deficiency (lane 4), and a cDNA negative control (lane 5) and three 10-fold dilutions of control cDNA (1×, 0.1×, and 0.01×) (lanes 6 through 8, respectively) were amplified with primers specific for VDJ-rearranged μ heavy chain and TdT, which was used as a control to demonstrate equal concentrations of pro-B cell transcripts.

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8. The sequence flanking each exon of BLNK has been deposited in GenBank (accession numbers AF180740 through AF180756). The primers used to amplify each exon are available upon request. The conditions used in SSCP are as described in work by M. E. Conley, M. E. Fitch-Hilgenberg, J. L. Cleveland, O. Parolini, and J. Rohrer [*Hum. Mol. Genet.* **3**, 1751 (1994)].
9. The following primers were used to amplify BLNK exon 1 and the associated flanking sequence for both the SSCP analysis and cloning of this region of the gene: 5′-GAACTGCTGACGTGACCA-3′ (5′ untranslated region of exon 1) and 5′-CCCTAAAAGCT-CAGTCCAC-3′ (intron 1). The products of two independent PCR reactions were cloned and sequenced.
10. DNA samples from five individuals demonstrated altered migration in comparison to the wild-type pattern. Sequencing showed a T to A substitution at the +113 position in intron 1 in three people, a G to A substitution at the +121 position in intron 1 in one individual, and an A to G substitution 5 base pairs upstream of the start codon.
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12. The primers used to amplify BLNK cDNA were from the 5′ untranslated region [shown in (9)] and 5′-GTCGCTGTCAAAGTCATCGGA-3′ from exon 4. The Btk-specific primers were from exons 6 and 8 of that gene. The primers used to amplify TdT, λ5, VDJ-μ, and glyceraldehyde phosphate dehydrogenase (GAPDH) were as reported in (7).
13. The father had 6% and the mother had 8% CD19$^+$ B cells (normal is 5 to 22%). Serum IgG in the father was 1110 mg/dL IgA was 337 mg/dL and IgM was 93 mg/dL. The mother's IgG was 1060 mg/dL IgA was 115 mg/dL and IgM was 127 mg/dL.
14. Bone marrow mononuclear cells were stained with antibody to CD19 conjugated to phycoerythrin (PE) (Dako, Carpinteria, CA), antibody to CD34 conjugated to peridinin chlorophyll protein (PerCP) (Becton-Dickinson, San Jose, CA), and polyclonal antibodies against human light chains conjugated to fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, AL). Antibodies to nuclear TdT (Supertechs, Bethesda, MD) and cytoplasmic μ heavy chains (Southern Biotechnology Associates) conjugated to FITC and PE, respectively, were applied after cell permeabilization with OrthoPermeafix (Ortho Diagnostics, Raritan, NJ). Monoclonal antibody to BLNK (of IgG2a isotype) was generated against a glutathione S-transferase fusion protein encoding amino acids 4 through 205, as described in (7). This antibody was used in combination with an antibody to CD19 of IgM class (BLY3) (Research Diagnostics, Flanders, NJ), with fluorochrome-conjugated secondary antibodies specific for murine IgG and IgM. Antibody to BLNK was applied after cell permeabilization with OrthoPermeafix. Isotype-matched nonreactive antibodies were from Becton-Dickinson. Immunofluorescence staining was analyzed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson).
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Perforin Gene Defects in Familial Hemophagocytic Lymphohistiocytosis

Susan E. Stepp,[1] Rémi Dufourcq-Lagelouse,[2] Françoise Le Deist,[2,3] Sadhna Bhawan,[1] Stéphanie Certain,[2] Porunelloor A. Mathew,[4] Jan-Inge Henter,[5] Michael Bennett,[1] Alain Fischer,[2,3] Geneviève de Saint Basile,[2]*† Vinay Kumar[1]*

Familial hemophagocytic lymphohistiocytosis (FHL) is a rare, rapidly fatal, autosomal recessive immune disorder characterized by uncontrolled activation of T cells and macrophages and overproduction of inflammatory cytokines. Linkage analyses indicate that FHL is genetically heterogeneous and linked to 9q21.3–22, 10q21–22, or another as yet undefined locus. Sequencing of the coding regions of the perforin gene of eight unrelated 10q21–22–linked FHL patients revealed homozygous nonsense mutations in four patients and missense mutations in the other four patients. Cultured lymphocytes from patients had defective cytotoxic activity, and immunostaining revealed little or no perforin in the granules. Thus, defects in perforin are responsible for 10q21–22–linked FHL. Perforin-based effector systems are, therefore, involved not only in the lysis of abnormal cells but also in the down-regulation of cellular immune activation.

FHL is a hemophagocytic lymphohistiocytic disorder in which previously healthy young children present with fever, splenomegaly, hepatomegaly, pancytopenia, coagulation abnormalities, neurological abnormalities, and high serum concentrations of interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α). Accumulation of activated macrophages and lymphocytes, mainly CD8$^+$ human lymphocyte antigen DR$^+$ Fas$^-$ T cells, as well as hematophagocytosis in the bone marrow, spleen, liver, lymph nodes, and central nervous system, dominate the pathology (*1–3*). Defective T and natural killer (NK) cell cytotoxicity is consistently reported (*4, 5*). We hypothesized that the primary inherited defect in FHL could be a failure of cytolytic lymphocyte function and that this, together with childhood infections (*6, 7*), induces the fatal immune deregulation of FHL.

The gene encoding perforin, an important mediator of lymphocyte cytotoxicity, has been mapped to 10q22 (*8*), near one of the previously identified FHL-linked loci (*9, 10*). Thus, perforin deficiency may play a role in the pathogenesis of FHL. Unlike patients with FHL, perforin knockout mice are generally healthy when maintained in a pathogen-free, controlled environment. However, when infected with lymphocytic choriomeningitis virus (LCMV), similar CD8$^+$ T cell–, IFN-γ–, TNF-α–dependent immunopathology and mortality are seen (*11, 12*).

We first confirmed the presence of perforin (PRF1) in the candidate region by polymerase chain reaction (PCR) screening a partial yeast artificial chromosome (YAC) contig covering the FHL region on chromosome 10 using prim-

[1]Department of Pathology and the Graduate Program in Immunology, University of Texas Southwestern Medical School, Dallas, TX 75235, USA. [2]Unité de Recherches sur le Développment Normal et Pathologique du Système Immunitaire INSERM U429, 75015 Paris, France. [3]Unité d'Immunologie et d'Hématologie Pédiatrique, Hôpital Necker-Enfants Malades, 75015 Paris, France. [4]Department of Molecular Biology and Immunology, University of North Texas Health Science Center, Fort Worth, TX 76107, USA. [5]Child Cancer Research, Karolinska Institutet, Department of Pediatric Hematology and Oncology, Karolinska Hospital, Stockholm S-17177, Sweden.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: sbasile@necker.fr

Fates of human B-cell precursors

Tucker W. LeBien

Development of mammalian B-lineage cells is characterized by progression through a series of checkpoints defined primarily by rearrangement and expression of immunoglobulin genes. Progression through these checkpoints is also influenced by stromal cells in the microenvironment of the primary tissues wherein B-cell development occurs, ie, fetal liver and bone marrow and adult bone marrow. This review focuses on the developmental biology of human bone marrow B-lineage cells, including perturbations that contribute to the origin and evolution of B-lineage acute lymphoblastic leukemia and primary immunodeficiency diseases characterized by agammaglobulinemia. Recently described in vitro and in vivo models that support development and expansion of human B-lineage cells through multiple checkpoints provide new tools for identifying the bone marrow stromal cell–derived molecules necessary for survival and proliferation. Mutations in genes encoding subunits of the pre-B cell receptor and molecules involved in pre-B cell receptor signaling culminate in X-linked and non–X-linked agammaglobulinemia. A cardinal feature of these immunodeficiencies is an apparent apoptotic sensitivity of B-lineage cells at the pro-B to pre-B transition. On the other end of the spectrum is the apoptotic resistance that accompanies the development of B-lineage acute lymphoblastic leukemia, potentially a reflection of genetic abnormalities that subvert normal apoptotic programs. The triad of laboratory models that mimic the bone marrow microenvironment, immunodeficiency diseases with specific defects in B-cell development, and B-lineage acute lymphoblastic leukemia can now be integrated to deepen our understanding of human B-cell development. (Blood. 2000; 96:9-23)

Introduction

Development of mature blood cells from lymphohematopoietic progenitors is a complex process governed by sequential changes in gene expression and external cues emanating from lymphohematopoietic microenvironments, such as fetal liver and bone marrow (BM). The last decade has witnessed dramatic progress in elucidating the molecular mechanisms that govern blood cell development. Mice with alterations in gene content (transgenics, knockouts, knockins) have been extraordinarily useful in elucidating the role of transcription factors, cytokines, and cytokine receptors in blood cell development. This review focuses on the developmental biology of human BM B-lineage cells and on perturbations in development that can contribute to the progression of B-lineage acute lymphoblastic leukemia (ALL) and immunodeficiency diseases characterized by agammaglobulinemia. My objective is to provide an update on key issues in human B-cell development and, where appropriate, compare and contrast B-cell development in mouse and human. The discussion of B-lineage ALL and immunodeficiency diseases will consider the developmental biology of these diseases as they constitute a deviation from normal programs.

The terminology used in this review is largely consistent with the terminology used by other laboratories studying human B-cell development. Pro-B cells are those B-lineage cells that express cell-surface CD19 but do not express cytoplasmic or cell-surface μ heavy chains (HCs). Pre-B cells express cell-surface CD19 and cytoplasmic μHCs, and variably express cell-surface μHCs associated with surrogate light chains (ψLCs)—ie, the pre-B cell receptor (pre-BCR). Immature B cells express cell-surface CD19 and cell-surface μHCs associated with κ or λLCs—ie, the B-cell receptor (BCR). B-cell precursors include all B-lineage cells prior to immature B cells expressing the BCR.

Sites of B-cell development

Human B-lineage cells are present in multiple tissue sites in early fetal development. However, from midgestation through the eighth decade of life, the BM is the exclusive site of B lymphopoiesis. Pre-B cells are present in 7- to 8-week gestational age fetal liver[1] and 10-week gestational age fetal omentum.[2] A thorough analysis of 18- to 20-week fetal tissues revealed that B-cell development is multifocal: $CD19^+$/surface μHC^- B-cell precursors and $CD19^+$/surface μHC^+ immature B cells are present in BM, liver, lung, kidneys, and spleen.[3] The frequency of B-cell precursors as a percentage of the total lymphoid cell pool is much higher in fetal BM compared with adult BM.[3,4] Adult BM also differs from fetal BM by the presence of recirculating $CD19^+$/surface μ, δ HC^+ mature B cells in the former.[3] Similar levels of recombinase-activating gene (RAG)–1, RAG-2, and terminal deoxynucleotidyl transferase (TdT) are detectable by reverse transcriptase–polymerase chain reaction (RT-PCR) in pro-B cells from 18-week fetal BM and 62-year adult BM, underscoring the functional integrity of BM B-cell development throughout life.[3] It is noteworthy that recent studies of T-cell development indicate that T-cell receptor (TCR) gene rearrangements actively occur in thymocytes from individuals

From the Department of Laboratory Medicine and Pathology, University of Minnesota Cancer Center, and Center for Immunology, University of Minnesota, Minneapolis.

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Reprints: Tucker W. LeBien, University of Minnesota Cancer Center, 420 Delaware St SE, Box 806 Mayo, University of Minnesota, Minneapolis, MN 55455; e-mail: lebie001@tc.umn.edu.

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in their sixth decade of life.[5] Thus, ongoing development of B and T lymphocytes throughout life complements the existence of memory B and T lymphocytes in maintaining a functional immune system.

Developmental stages of B-lineage cells

Analysis of gene expression in developing lymphoid cells can be accomplished by multiparameter flow cytometry, immunohistochemistry/fluorescence microscopy, and RT-PCR. Immunologic phenotyping of B-lineage ALL using monoclonal antibodies (mAbs) and flow cytometry has been conducted in many laboratories, and it is not my intent to summarize the many published reports. The reader is referred elsewhere for an in-depth review.[6]

Although lymphoid progenitors are the descendants of hematopoietic stem cells (HSCs) with the capacity to develop into all lymphohematopoietic lineages, the earliest populations of lineage-restricted lymphoid progenitors are poorly characterized. Figure 1 shows potential relationships between so-called common lymphoid progenitors (CLPs) and progenitors with increased fitness to form specific lymphoid lineages. The CLP is defined as a progenitor with the capacity to develop into T, B, or natural killer (NK) cells, but little or no capacity to develop into nonlymphoid lineages, such as myeloid/erythroid cells. A cell with the developmental potential of a CLP has been included in the blood cell developmental schemes of hematology textbooks for many years. However, data supporting its existence have only recently been published.[7-10] Galy and colleagues used fluorescence-activated cell sorting (FACS) to purify CD34$^+$/CD10$^+$/CD45RA$^+$ BM progenitors that do not express T, B, or NK lineage surface markers (ie, CD2, CD4, CD8, CD16, CD19, CD20, and CD56). A battery of in vitro assays and the severe combined immunodeficiency (SCID)–hu (human) mouse were employed to demonstrate that CD34$^+$/CD10$^+$/CD45RA$^+$ progenitors are capable of developing into B, T, NK, and lymphoid dendritic cell (DC) lineages, but not myeloid/erythroid lineages.[7]

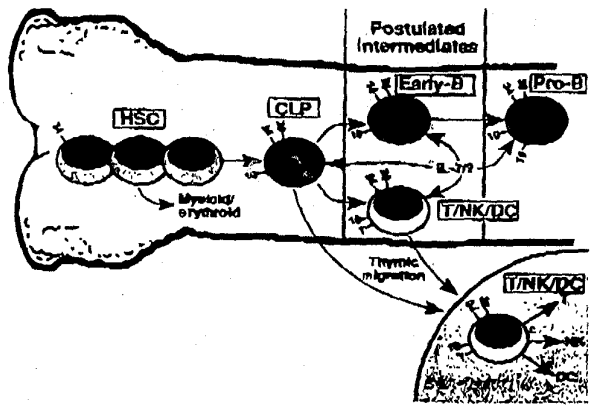

Figure 1. Developmental relationship between hematopoietic stem cells (HSCs), common lymphoid progenitors (CLPs), and putative early-B or T/NK/dendritic cell (DC) progenitors. HSCs include all primitive CD34$^-$/lineage$^-$ hematopoietic developmental stages prior to the CLP, shown schematically as 3 cells. Arrows with solid lines indicate developmental flow culminating in increased lineage restriction. Dashed arrows indicate possible cellular targets of IL-7 signaling or an unknown (?) ligand. Numbers on the cell surface indicate CD antigens useful in distinguishing the developmental compartments. Although not shown in this figure, the 3 reports that described the cell surface phenotype of CD19$^-$ lymphoid progenitors revealed considerable heterogeneity.[7,8,13] For example, CD7 and CD33 were detected on a minority of the lymphoid progenitors in each study.[7,8,13] There is no known surface marker that distinguishes the CLP from the early-B cell. It is also likely that IL-7R expression and signaling vary both within and between the lymphoid progenitor compartments.

However, this study did not clarify whether T- and B-lineage cells are derived from a single progenitor. A subsequent study by Ryan and colleagues showed that CD34$^+$/CD19$^-$ lymphoid progenitors expressing the interleukin 7 receptor (IL-7R) (CD127) gave rise to CD19$^+$ cells in a colony-forming assay.[9] The CD34$^+$/IL-7R$^+$ lymphoid progenitors were uniformly TdT$^+$, and the majority expressed CD10. RT-PCR analysis indicated the expression of RAG-1, immunoglobulin (Ig)β (CD79b), and the paired domain transcription factor PAX-5.[8] Ryan and colleagues did not assay for T, NK, or DC potential, but the CD34$^+$/IL-7R$^+$/CD19$^-$ population contained granulo-monocytic colony–forming units at a frequency 10-fold to 100-fold lower than IL-7R$^-$/CD19$^-$ cells.[9] A recent report indicated that CD34$^+$ BM cells expressing the CXCR4 chemokine receptor for stromal cell–derived factor-1 (discussed in more detail below) could develop into B- and T-lineage cells, but not myeloid/erythroid cells.[9] It seems likely that the 3 reports[7-9] described a human lymphoid progenitor with similar developmental potential. A population of IL-7R$^+$ adult murine BM cells are also developmentally restricted to become T, B, and NK cells[10] and may be the murine counterpart of the lymphoid progenitors isolated from human BM.[7-9]

The model in Figure 1 proposes that CLP can differentiate into 1 of 2 lymphoid progenitor intermediates: early-B cells or T/NK/DC tri-lineage cells. Early-B cells are characterized by the initiation of DJ_H rearrangements and the expression of B-lineage specific proteins such as VpreB and Igα (CD79a). Support for the existence of an early-B cell comes from reports showing that DJ_H rearrangements,[11,12] cytoplasmic Igα protein,[13] and VpreB protein[14] are present in CD19$^-$ lymphoid progenitors. The CLP could also differentiate into a T/NK/DC tri-lineage progenitor possibly defined by the acquisition of CD7. Either the CLP or the T/NK/DC progenitor could migrate to the thymus and undergo subsequent development.[15] It is unknown whether specific signals transduced by BM stromal cell–derived molecules can promote CLP differentiation into an early-B cell or a T/NK/DC progenitor.

Murine CLPs and possibly human CLPs are particularly sensitive to IL-7 stimulation. Signaling through the IL-7R is essential for the development of murine CLPs, although not by virtue of inducing self-renewal.[16] A role for IL-7 signaling in the development of human CD19$^+$ B-lineage cells from CD10$^-$/TdT$^+$/CD19$^-$ lymphoid progenitors has been demonstrated in vitro,[8] but whether IL-7 exerts an effect on CLPs and/or early-B cells is unknown. The relationship between signaling pathways activated following IL-7 stimulation of CLPs and the developmental fate of CLPs are unknown. IL-7 activation of phosphatidylinositol (PI)–3 kinase/protein kinase B activation is essential for survival/proliferation of human T-cell precursors, whereas IL-7 activation of STAT5 favors T-cell differentiation.[17] It will be interesting to determine whether PI-3 kinase, STAT5, or other IL-7 signaling pathways activated in human CLPs can lead to different developmental fates. The CLP—early-B cell step cannot *require* IL-7 (see below), and other cytokines or BM stromal cell–derived compensatory signals must be important.

Figure 2 shows the stages of human B-cell development and counterpart stages in murine B-cell development. Several classification schemes have been developed for the mouse,[18] but Figure 2 shows the A through E fractions originally described by Hardy and colleagues.[19] Figure 2 proposes the existence of an early-B cell (as discussed above) that does not express the cell surface protein CD19. Early-B cells have initiated DJ_H rearrangements and express cytoplasmic Igα (and to some degree Igβ) as well as VpreB proteins. I would emphasize that this is a tentative definition of an

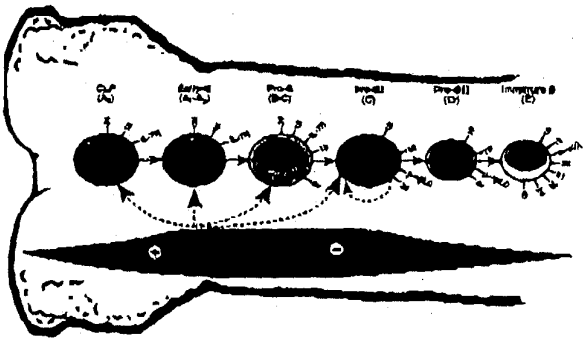

Figure 2. Stages in human B-cell development. Six stages beginning with the CLPs and culminating with immature B cells are shown as one model of B-cell development in human BM. The letters in parentheses represent an approximation of the counterpart stages in murine B-cell development, using the nomenclature of Hardy and colleagues.[18,20] Numbers on the cell surface indicate CD antigens frequently used to define the individual stages in human B-cell development. Patterns of gene expression inside the cells have been determined by RT-PCR and/or flow cytometry. Dashed arrows indicate possible cellular targets for positive (+) and negative (−) growth regulators/chemotactic factors produced by BM stromal cells. Cells in a particular developmental stage are not necessarily uniform in the expression of a specific receptor. For example, only 10% to 20% of the pre-BI plus the pre-BII cells express the μ-ψLC pre-BCR.

early-B cell, since no cell surface markers that could distinguish early-B cells from CLP have been reported. This early-B cell may be similar to the $CD19^-$ B-lineage fractions A_1 and/or A_2 recently described by Hardy and colleagues,[20] or the lin^-/c-kit^{lo} and lin^-/c-kit^- progenitors described by Payne and colleagues.[21] Human pro-B cells are a well-characterized population expressing CD10, CD34, and CD19.[22] The vast majority of pro-B cells express TdT,[22-24] and V-to-DJ_H rearrangements are easily detected.[11,12] However, single-cell PCR analysis indicated that a minority of $CD19^+/CD34^+$ pro-B cells have both HC alleles in germ-line configuration.[24] Thus, assigning the early-B cell population a DJ_H rearrangement status and the pro-B cell population a VDJ_H rearrangement status is probably an oversimplification. A difference of opinion exists as to whether $CD19^+/CD34^+$ pro-B cells express cytoplasmic μHC. Two groups have concluded that pro-B cells express neither cytoplasmic nor low cell-surface μHC.[24,25] In contrast, using FACS-purified $CD19^+/CD34^+$ pro-B cells, we can reproducibly detect cytoplasmic μHC in 5% to 10% of pro-B cells.[26] This result is consistent with the existence of readily detectable VDJ_H rearrangements in pro-B cells.[11,12] The human pro-B cell may correspond to Hardy fraction B ± fraction C, based on analysis of VDJ_H rearrangements.[24,27]

A functional VDJ_H rearrangement is essential for normal pro-B cell differentiation into the pre-BI compartment (Figure 2). Pro-B cells that fail to make a functional VDJ_H rearrangement undergo apoptosis and are probably phagocytized by BM macrophages. Pro-B cell differentiation into pre-BI cells is characterized by loss of CD34 and TdT, and acquisition of cytoplasmic μHC in more than 95% of the cells.[22-24] Similarly to the mouse,[18] human pre-B cells can be generally subdivided into large proliferating cells (designated pre-BI in Figure 2) and small postmitotic cells (designated pre-BII in Figure 2) on the basis of cell-cycle analysis.[24] The human pre-BI cells would partially overlap with Hardy fraction C.[19,20] Pre-BII cells are actively undergoing κLC rearrangements.[24] In general, κ rearrangement precedes λ rearrangement, and pre-BII cells that fail to make a functional κ rearrangement can proceed to rearrange the λLC locus. Interestingly, a very small percentage (approximately 1%) of immature B cells in human BM and peripheral blood express κ and λLC on individual cells.[28-30] This dual LC expression may reflect immature B cells undergoing receptor editing.

The pre-BCR and related structures

Mammalian B-lineage cells must traverse several critical checkpoints on the road to becoming functional antigen-specific B cells. The cell surface molecular complex appearing at a critical initial checkpoint is the pre-BCR. The pre-BCR is a complex of proteins consisting of μHC, ψLC, and the Igα/Igβ signal transducing heterodimer.[31] The mammalian ψLC consists of 2 proteins generally referred to as VpreB and λ5. The genes encoding these 2 proteins were originally discovered in the mouse (Melchers et al[32] and references therein) and their organization differs between mouse and human (Minegishi et al[33] and references therein) VpreB and λ5 proteins are noncovalently associated on the surface of B-cell precursors and together form a λLC-like structure. In turn, λ5 is covalently coupled to the C_H1 domain of μHC via a carboxy-terminal cysteine. Readers are referred to an earlier review for details on the original identification and characterization of the VpreB and λ5 genes and their encoded proteins.[32]

Analysis of the structure, expression, and function of the human pre-BCR has been facilitated by the development of mAbs against recombinant ψLC proteins.[34-39] The initial panel of mAbs made against the human ψLC was used to characterize cytosolic and cell-surface μHC/ψLC complexes and surface expression of ψLC.[34,40] A major conclusion in the original study[34] was that surface μHC/ψLC expression was restricted to the pre-B cell compartment (ie, $CD19^+/CD34^-$ cells in Figure 2); this suggests a critical role for the pre-BCR at a relatively late stage of B-cell development. Subsequent studies using other mAbs yielded conflicting results.[24,35,36] A major difference was the identification of normal and leukemic pro-B cells (ie, $CD19^+/CD34^-/\mu HC^-$) that stained with anti-VpreB mAb.[24,35,36] The low levels of cell-surface ψLC, differences in the subtype of the mAb (ie, IgM versus IgG1), and differences in epitope recognition by various anti-VpreB mAbs were among the explanations offered for the conflicting results.

A series of papers describing new antihuman VpreB mAbs[37-39] has provided some resolution to past discrepancies. The recent vintage of anti-VpreB mAbs includes mostly IgG1 subclass mAbs, thereby eliminating potential problems that can confound the use of IgM reagents. The anti-VpreB mAb produced by Wang and colleagues binds to the surface of pre-B cell lines, but only binds to pro-B cell lines following permeabilization.[37] As expected, normal $CD19^-$ human B-lineage cells coexpressed low levels of cell surface μHC and VpreB, and approximately 20% of the $CD19^+/VpreB^-$ cells were weakly $CD34^+$.[37] Interestingly, cytoplasmic $VpreB^-$ is expressed in $CD34^+/CD19^-$ lymphoid progenitors at a stage prior to V-to-DJ_H rearrangement,[37] possibly the early-B cells proposed in Figure 2. Tsuganezawa and colleagues generated mAbs that recognized human VpreB, human λ5, or an epitope expressed only on the assembled pre-BCR.[38] Their anti-VpreB mAb binds to the surface of pre-B ALL cell lines but not pro-B ALL cell lines.[38] However, cytoplasmic VpreB or λ5 were detected in the majority of pro-B ALL cell lines and freshly isolated pro-B ALL.[38] In contrast, the anti-VpreB mAb produced by Gauthier and colleagues[39] binds to the surface of some μ^- pro-B ALL cell lines.[25] One of these pro-B ALL cell lines (designated JEA2) was shown to express cell surface VpreB in association with poorly characterized molecules of approximately 105 to 130 kd. Interestingly,

cross-linking VpreB on the μ^- JEA2 pro-B ALL cell line led to an increase in Ca^{++} flux, suggesting that VpreB was one component of a putative signaling receptor on the surface of at least some pro-B ALL cells.[25] These authors also detected cell-surface VpreB on $CD19^+/CD34^+$ normal pro-B cells and used this data to argue for the existence of 2 distinct populations: $CD19^+/CD34^+/\mu HC^-$/$VpreB^+$ and $CD19^+/CD34^-/\mu HC^+/VpreB^+$. However, there is no biochemical evidence that VpreB is associated with a protein (or proteins) other than μHC on the surface of normal human B-cell precursors. We have used one of the anti-VpreB mAbs (VpreB8) made by Wang and colleagues[37] to analyze VpreB expression in fetal BM B-lineage cells. Our results indicate that VpreB is expressed on the surface of 5% to 10% of the B-cell precursors (ie, pro-B plus pre-BI plus pre-BII in Figure 2). Furthermore, within the $VpreB^+$ population, approximately 90% of the cells are $CD19^+/CD34^-$, and approximately 10% are $CD19^+/CD34^+$. Results from our laboratory show that $CD34^+/VpreB^+$ cells also express cell surface μHC (J. A. R. Pribyl, N. Shah, F. E. Bertrand, T. W. LeBien, 1999, unpublished data). Thus, we believe that most, if not all, $CD19^+/CD34^+/VpreB^+$ normal B-lineage cells express the conventional pre-BCR. Since the vast majority of surface $VpreB^+$ cells are $CD34^-$, I show the expression of the pre-BCR on pre-BI and pre-BII cells only (Figure 2). The $VpreB^+$ cells that weakly express CD34 could be developmentally more similar to pre-BI cells than pro-B cells, but this has not been tested. The vast majority of B-lineage ALLs express cytoplasmic or surface VpreB,[25,38] probably reflecting the general phenotypic similarity between normal and leukemic B-cell precursors.

The importance of the ψLC to normal B-cell development was first elucidated in a classic study demonstrating that mice with a targeted disruption in the λ5 locus exhibit a block at the pro-B to pre-B transition.[41] The block in B-cell development probably occurs because cells at this transition fail to receive a positive selection signal through the pre-BCR. However, the block is not absolute since the number of B cells in secondary lymphoid tissues gradually increases over time, probably owing to the emergence of B cells that rearranged κLC genes prior to μHC genes.[42,43] The importance of the ψLC in human B-cell development has been underscored by the discovery of an agammaglobulinemia patient with mutations in both λ5 alleles[33] (see below). Immunologic analysis of this single patient indicated a disruption in B-cell development more severe than what occurs in λ5-deficient mice. Since only a single patient with a mutation in the λ5 locus has been described to date, it is unclear whether the gradual recovery of B cells observed in λ5-deficient mice would occur in humans.

How does the pre-BCR perform its critical role at the pro-B—pre-B transition? Despite heuristic appeal, the notion that the pre-BCR functions as a receptor for a specific ligand in the BM or fetal liver microenvironment has not been supported by experimental evidence. What has become clear is that only about one half the μHC proteins encoded by functional VDJ_H rearrangements are capable of pairing with ψLC in the mouse.[44-46] Circumstantial evidence suggests that a similar type of preferential pairing of μHC and ψLC also occurs in human pre-B cells.[47,48] This pairing is essential for pre-BCR assembly and expression on the cell surface. Formation of the pre-BCR heralds a sequence of events, including (1) suppression of RAG-1/RAG-2 expression to ensure allelic exclusion at the μHC locus, (2) a rapid burst of proliferation in cells expressing the pre-BCR, and (3) reexpression of RAG-1/RAG-2 and initiation of LC gene rearrangement. Are there separable roles for ψLC and μHC in pre-BCR function? This is controversial. Shaffer and Schlissel reported that transgenic mice expressing a truncated murine μHC (incapable of pairing with ψLC) could still transduce signals leading to changes in surface markers, transcription, and retargeting of the recombinase ensemble in B-lineage cells.[49] The authors concluded that the ψLC functions as a chaperone to facilitate pre-BCR assembly and expression, but plays no direct role in signal transduction. They also argued that the capacity of truncated μHC to mediate B-cell differentiation in the absence of ψLC ruled against the pre-BCR/ligand model. However, truncated μHC may undergo enhanced aggregation compared with full-length μHC, thereby leading to increased constitutive signaling.[50]

Pre-BCR cross-linking in vitro initiates signaling events, including Ca^{++} flux and protein tyrosine kinase activation.[51,52] How could this occur in vivo in the absence of an external cross-linking ligand? Elegant recent studies have provided new insight into the complexity of pre-BCR subunit protein-protein interactions involving μHC, VpreB, and λ5.[39,53] These studies suggest mechanisms to explain pre-BCR assembly, V_H repertoire selection, and cell signaling. We might assume that signaling through the pre-BCR minimally requires the dimerization/aggregation of at least 2 distinct pre-BCR molecular complexes on the cell surface. With the realization that the ψLC is a non–transmembrane-spanning polypeptide complex disulfide-linked to the μHC and exhibiting VpreB-V_H interactions, it seems possible (as proposed by Melchers[54]) that the ψLC itself could assume a ligand function for the μHC.

Other protein complexes with potentially similar functions to the pre-BCR have been described. Murine pro-B cell lines express the ψLC associated with several proteins ranging from 65 to 200 kd, including a predominant protein of 130 kd.[55] These pro-B cell lines do not express μHC.[55] The protein or proteins associated with ψLC in these cells have been occasionally referred to as the surrogate HC. A recent study using a human μ^- pro-B leukemic cell line (JEA2) showed that VpreB is noncovalently associated with a p105 and possibly several other proteins on the cell surface.[25] However, there is no evidence that VpreB associates with p105 (or any other protein other than μHC) on normal human B-cell precursors. The identity of the murine and human ψLC-associated surface proteins is unknown. A novel complex that has been referred to as the "calnexin pre-BCR"[56] was recently described by Nagata and colleagues.[57] The calnexin pre-BCR consists of Igα/Igβ noncovalently associated with the molecular chaperone calnexin, and was detected on the surface of pro-B cell lines and early B-lineage cells from RAG-2–deficient mice.[57] Cross-linking Igβ on these cells induced the tyrosine phosphorylation of syk, ERK, and PI-3 kinase in vitro, and pro-B to pre-B cell differentiation in vivo. A potential μ-independent role for Igβ was discovered when mice with a targeted disruption of the Igβ gene were shown to exhibit a block in B-cell development prior to V-to-DJH rearrangement.[58] These results[57,58] suggest a role for $Ig\beta^+/\mu HC^-$ molecular complexes in the earliest stages of murine B-cell development.

The IL-7 story

An unresolved issue in studies of human B-cell development is the identity of the molecule(s) essential for the growth of normal B-cell precursors. Much has been written of IL-7, and some historical perspective is warranted. Following the initial cloning and characterization of IL-7 from murine BM stromal cells more than 10 years ago,[59] IL-7 was shown to be crucial for the proliferation and development of murine B-cell precursors. IL-7 has been cast as a survival, proliferation, or differentiation factor depending upon the experimental system being employed.[60,61] Mechanistic insight has

been gleaned from studying the effect of single amino acid substitutions on IL-7R α chain function. Corcoran and colleagues showed that a Y-to-F mutation at amino acid residue 449 abrogated the capacity of B-cell precursors to undergo IL-7–induced proliferation through a PI-3 kinase–dependent pathway.[62] Interestingly, functional studies of the IL-7R α chain harboring this mutation uncovered a novel signaling pathway (PI-3 kinase independent?) that triggered IgH rearrangements and subsequent B-cell differentiation.[62] Recent studies from the same group indicated that IL-7R signaling can alter recombinase accessibility of 5′ V_H genes.[63] The criticality of IL-7 for normal murine B-cell development has been elucidated in gene-targeted mice. Targeted disruptions in the genes encoding IL-7,[64] the IL-7R α chain,[65] the γ_c subunit of the receptors for IL-2, 4, 7, 9, and 15,[66,67] and the Jak3 tyrosine kinase[68,69] all lead to severe impairment in B-cell development. Thymocyte and T-cell development are also impaired, reflecting the multiple actions these 5 cytokines exert on lymphopoiesis. IL-7 is, however, not the only cytokine implicated in the regulation of murine B-cell development. Kincade and colleagues have suggested that at least 16 distinct stromal cell products can exert positive effects on murine B-cell development.[61] One recent addition to the list is thymic stromal lymphopoietin (TSLP). TSLP was originally isolated from a murine thymic stromal cell line.[70] TSLP reportedly has the capability to replace IL-7 in supporting murine B-cell development in vitro[71] and promote the development of surface IgM^+ immature B cells from surface IgM^- precursors.[72] Interestingly, the TSLP receptor complex consists of the IL-7R α chain and a second subunit distinct from the γ_c. Furthermore, TSLP induced the activation of STAT5 but not any of the known Jak kinases.[72] Whether IL-7 and TSLP work in a hierarchical or cooperative manner in regulating murine B-cell development is unknown. Human TSLP has been cloned (S. Lyman, PhD, Immunex, written communication, May 1999), but there are no published reports on its bioactivity on human B-cell precursors.

The role of IL-7 in human B-cell development is strikingly different from its role in murine B-cell development. In fact, there is a widespread misconception that IL-7 stimulates the proliferation of human B-cell precursors. Part of the difficulty in determining IL-7 effects can be attributed to differences in the assay systems employed and the biological endpoints measured. Initial studies by us[73] and others[74,75] demonstrated that recombinant human IL-7 could exert weak proliferative effects on normal human B-cell precursors in vitro. However, it was difficult to exclude the possibility that IL-7 was simply enhancing survival. A stronger effect of IL-7 was observed when normal B-cell precursors were cultured on human BM stromal cells.[26,73,75,76] Under these conditions, $CD19^+$ B-lineage cell numbers increased by 10-fold to 20-fold over 2 to 3 weeks in vitro, but underwent rapid cell death thereafter.

Biological insight into IL-7 function in human B-cell development has been gleaned from congenital immunodeficiency patients. Patients with X-linked severe combined immunodeficiency (XSCID) have mutations in the γ_c subunit of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. XSCID patients have severe defects in development of T and NK cells, but have normal or even elevated numbers of peripheral blood B cells.[77] Furthermore, immunodeficiency patients with autosomal recessive mutations in the Jak3 tyrosine kinase exhibit a developmental phenotype indistinguishable from XSCID, including normal numbers of peripheral blood B cells.[78,79] Likewise, 2 patients with mutations in the IL-7R α chain that led to defective expression also had normal numbers of peripheral blood B cells.[80] These collective experiments of nature clearly indicate that IL-7 signaling is not essential for at least the numerically normal development of human B lymphocytes. My laboratory used a human BM stromal cell culture system to show that HSCs could develop into B-lineage cells independently of IL-7 stimulation.[81] Our results are in accord with B-cell development occurring in patients with XSCID. However, extensive proliferation of the pro-B cell compartment does not occur,[81] and it is presumptuous to assume that this in vitro model completely mimics B-cell development occurring in patients with mutations in the IL-7 pathway.

Although not essential for human B-cell development, IL-7 does transduce signals that lead to specific changes in gene expression. Proliferation of $CD19^+/CD34^+$ pro-B cells on human BM stromal cells is enhanced by inclusion of exogenous IL-7.[26] IL-7 stimulation induces a specific increase in cell-surface CD19 on human pro-B cells[82,83] and a decrease in RAG-1, RAG-2, and TdT messenger RNA (mRNA) levels.[83] There may be physiologic relevance to these results. For example, IL-7 expression in situ has been detected by RT-PCR analysis of human BM biopsies.[84] The identity of the human BM cell producing IL-7 in vivo is unknown, although small amounts (less than 2 pg/mL) of IL-7 can be detected in supernatants from vascular cell adhesion molecule-1 (VCAM-1) $(CD106)^+$ BM stromal cells in vitro.[76,81,85] Similarly, purified $VCAM\text{-}1^+$ murine BM reticular cells express cytoplasmic IL-7 protein.[86]

The complete identity of the human BM stromal cell–derived molecule (or molecules) that transduce signals essential for human B-cell development (including the counterpart of the murine IL-7 "signal") is unknown. Part of the difficulty in identifying an IL-7 alternative is that IL-7 could act on at least 3 distinct stages of lymphoid cell development: CLPs, early-B cells, and pro-B cells (Figure 2). The cell-cycle disposition and self-renewal capacity of these compartments have not been determined. However, we do know that human $CD19^-$ progenitors[8] and pro-B cells[26] are IL-7 responsive. Figure 3 shows several cytokines that could regulate human B-cell development. The cytokine-responsive target cells would include CLPs, early-B cells, and pro-B cells. The BM stromal cell molecules that regulate development could be secreted or cleaved from the stromal cell surface. The secreted or cleaved products could in turn be bound to stromal cell proteoglycans such as heparan sulfate proteoglycans (HSPGs). Several candidates

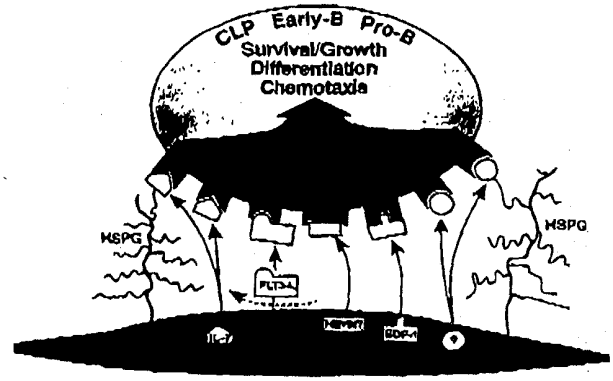

Figure 3. BM stromal cell–derived molecules that could transduce survival/growth, differentiation, or chemotactic signals to CLP, early-B, or pro-B cells. HSPG indicates heparan sulfate proteoglycan. The dashed arrow indicates that the membrane-bound form of Flt3 ligand is cleaved at the stromal cell surface. The question mark indicates the unknown growth factor that could bind to HSPG. IL-7 and the unknown growth factor are shown binding directly to their cognate receptors, or binding HSPG followed by binding to their cognate receptors.

come to mind. Namikawa and colleagues reported that a combination of IL-7, IL-3, and Flt3 ligand is superior to IL-7 alone in supporting human pro-B cell growth on murine BM stromal cells.[87] My laboratory has confirmed their observation using human BM stromal cells (J.A.R. Pribyl and T.W. LeBien, unpublished observations, February 1999). Flt3 ligand is produced by BM stromal cells[88,89] (also in our unpublished observations, February 1999) and could potentially stimulate similar cellular compartments and transduce similar signals to IL-7. Oritani and Kincade developed a cloning strategy to identify murine BM stromal cell gene products that bind to murine pre-B cells.[90] One of the molecules identified was a secreted extracellular matrix glycoprotein designated SC1/ECM2.[90] Soluble and immobilized SC1/ECM2 enhance the growth of IL-7–dependent murine pre-B cells,[90,91] but the mechanism of enhancement is unknown. Interestingly, the carboxy terminus of SC1/ECM2 has high amino acid sequence homology to osteonectin/SPARC,[92] which reportedly can bind cytokines, such as platelet-derived growth factor.[93] A human homologue of SC1/ECM2 (designated hevin) has been cloned from high endothelial venules,[94] but it is not known whether hevin has any effect on the growth of human B-cell precursors. HSPGs play a critical role in "presenting" cytokines to survival/growth factor receptors on lymphohematopoietic cells. HSPGs expressed on murine B-lineage and BM stromal cells can bind IL-7 and enhance the growth of IL-7–dependent murine pre-B cell lines.[95] Furthermore, heparan sulfate is important for cytokine-mediated expansion of human long-term-culture–initiating cells.[96] Therefore, a currently unknown molecule produced by BM stromal cells could bind to HSPGs and mediate survival/growth of human B-cell precursors (Figure 3).

A BM stromal cell product shown in Figure 3 that potentially provides a unique function in B-cell development is the chemokine stromal cell–derived factor-1 (SDF-1). Mice with targeted disruptions in the genes encoding SDF-1 and its receptor CXCR4 exhibit perinatal mortality owing to perturbations in organ vascularization and lymphohematopoiesis.[97-99] B lymphopoiesis and myelopoiesis are severely impaired,[97-99] and a recent study concluded that a functional CXCR4 receptor is essential for retention of B-cell precursors in the BM microenvironment.[100] CXCR4 has a complex pattern of expression on $CD34^+$ lymphohematopoietic cells and $CD19^+$ B-lineage cells.[9,100-102] CXCR4 is expressed at all stages of B-cell development, but $CD19^+/CD34^-/LC^-$ pre-B cells and mature B cells express higher levels than $CD19^+/CD34^+$ pro-B cells.[102,103] Interestingly, SDF-1–mediated signaling pathways leading to calcium mobilization and chemotaxis are more rigorously activated in less mature B-lineage cells expressing lower levels of cell surface CXCR4.[102,103] Thus, BM SDF-1 may trigger signaling pathways that regulate chemotaxis of B-cell precursors to "preferred sites" of proliferation within the stromal cell milieu. It is noteworthy that SDF-1 is expressed in human fetal liver biliary ductal plate epithelial cells, in apposition to lymphoid cells expressing VpreB.[104]

Models of human B-cell development

Establishment and characterization of in vitro culture systems that at least partially mimic the in vivo BM microenvironment have been extremely important for advancing our understanding of human B-cell development. Progress in this area has been facilitated by the use of BM stromal cells as a supportive microenvironment. Adherence of B-cell precursors to BM stromal cells is essential for normal murine and human B-cell development (reviewed in Kincade et al,[61] Jarvis et al,[105] and Ryan et al[106]). Binding of very late antigen–4 (VLA-4) (CD49d/CD29) expressed on human B-cell precursors to VCAM-1 on human BM stromal cells is the primary molecular interaction that facilitates adhesion of these 2 cell types.[107-109] Cytokines can regulate levels of BM stromal cell surface VCAM-1, thereby influencing the capacity of these cells to support B-cell precursor adhesion.[108] There is substantial evidence that cross-linking VLA-4 with VCAM-1 or the CS-1 domain of fibronectin can trigger a protein tyrosine kinase cascade in B-lineage cells. However, there is no evidence that VLA-4 triggers a reciprocal activation of VCAM-1 culminating in a signal transduced in BM stromal cells. Lymphoid cell contact with human BM stromal cells can transduce signals leading to protein tyrosine kinase activation[110] and tyrosine phosphorylation of focal adhesion kinase, paxillin, and ERK2.[111] This signaling pathway is independent of VCAM-1.[110] To what degree these bi-directional (B-cell precursor ↔ BM stromal cell) signaling events might influence B-cell developmental fates in vivo is unknown.

Prompted by our success in establishing in vitro human BM stromal cell culture conditions that support the adhesion[108] and short-term growth[26,73] of B-cell precursors, we asked whether a more expanded model of B-cell development could be established. In an effort to foster physiologic relevance, we FACS-purified fetal BM $CD34^{++}/CD19^-$ HSC and plated them onto third-passage, nontransformed human fetal BM stromal cells.[81] $CD34^{++}/CD19^-$ HSCs underwent commitment and differentiation into the B-lineage over a 3-week period.[81] A hierarchy of developmental changes consonant with B-cell development in vivo occurs in this in vitro model, including (1) loss of CD34 expression, (2) a continuum of increase in cell surface CD19, (3) emergence of cytoplasmic μHC^- pre-B cells, including some expressing the cell-surface pre-BCR, and (4) emergence of immature B cells expressing μ/κ or μ/λ BCR. As discussed above, several lines of evidence rule out a mandatory role for IL-7 in this culture system.[81] The fact that $CD34^{++}/CD19^-$ HSC can differentiate all the way to immature B cells demonstrates that human fetal BM stromal cells can provide the developmental cues necessary to traverse the major checkpoints defined by rearrangement of HC and LC genes. This human BM stromal cell culture does not support a dramatic numerical expansion of any specific compartment of B-lineage cells, probably attributable to the exclusion of fetal bovine serum and exogenous cytokines. It is also conceivable that the BM stromal cells in this culture (which are exclusively adventitial reticular/fibroblastlike cells by third passage) do not represent the totality of BM stromal cell components essential for optimal proliferation in vivo.

At the time we were developing our human BM stromal cell culture, Rawlings and his colleagues were developing an in vitro culture using the murine S17 stromal cell line.[112] They originally showed that enriched $CD34^+$ cord blood cells would develop into $CD19^+$ B-lineage cells after 3 to 4 weeks.[112] The $CD19^+$ B-lineage cells were at a very early stage of B-cell development since bulk culture analysis by Southern blotting indicated no rearrangements at the IgH locus.[112] These $CD19^+$ B-lineage cells could be expanded following transfer to fresh S17 stromal cells, but did not proliferate following stimulation with IL-7 or stem cell factor (SCF). A follow-up report showed that inclusion of Flt3 ligand enhanced the development of $CD19^+$ B-lineage cells by twofold to threefold.[113] We have recently shown that inclusion of Flt3 ligand at the initiation of our human BM stromal cell culture also enhances the development of $CD19^+$ B-lineage cells (J.A.R. Pribyl and T.W.

LeBien, unpublished observations, February 1999), providing additional support for a role of Flt3 ligand in human B-cell development (Figure 3).

Given the differences in the tempo and degree of B-cell development in the 2 models, we conducted a side-by-side comparison of murine S17 stromal cells and human fetal BM stromal cells.[114] When human fetal BM CD34+ HSCs were cultured on human fetal BM stromal cells or human skin fibroblasts, robust differentiation to the immature B-cell stage occurred within 3 weeks.[114] In contrast, CD19+ B-lineage cells emerging on S17 stromal cells within the same time frame had twofold to fourfold higher levels of cell-surface CD19, but no cells expressing the BCR.[114] Human and murine S17 stromal cells therefore differ in their capacity to support human B-cell differentiation under the conditions in which we compared them. The identity of the soluble or membrane-bound stromal cell molecules important in both cultures is unknown. When CD34+ cord blood HSCs are cultured on S17 stromal cells for 6 to 8 weeks, small numbers of cytoplasmic and surface μHC+ cells can be detected.[115] Furthermore, transferring these 6- to 8-week cultures onto CD40 ligand (CD154+) fibroblasts supplemented with IL-4 and IL-10 results in terminal human B-cell differentiation to Ig-secreting cells.[115] Murine stromal cell lines other than S17 also support the development of CD19+ human B-lineage cells from CD34+ cord blood HSCs.[116-119] Two of these studies showed that a combination of SCF and granulocyte colony–stimulating factor would enhance the outgrowth of B-cell precursors.[117,118]

The nonobese diabetic-severe combined immunodeficient (NOD-SCID) mouse[120] has become a popular tool for studying engraftment and development of human HSCs in vivo (for review, see Greiner et al[121]). The CD34++/CD38− HSC that engrafts in NOD-SCID mice has been designated the SCID-repopulating cell.[122] Development of CD19+ B-lineage cells from human CD34+ BM or cord blood HSCs transplanted into NOD-SCID mice has been reported by several groups.[122-126] The degree of human B-cell differentiation was variable in these studies, although spleen and peripheral blood B cells expressing surface μHC and κ or λLC were detected in 2 studies.[124,125] These results indicate that xenogeneic factors produced in NOD-SCID mice can promote and support multiple stages of human B-cell development. The murine BM appears to be the primary site of engraftment by human CD34+ HSCs. It follows that commitment into the human B-lineage and traversal through the pre-BCR and BCR checkpoints is likely to occur in murine BM, although this has not been directly demonstrated.

How can these in vitro and in vivo models of human B-cell development be further refined? None of the in vitro BM stromal cell cultures described thus far fully recapitulate the complex microenvironment in which B cells develop. For example, the adventitial reticular (fibroblastlike) adherent cell in the human BM stromal cell culture is only 1 component of the BM microenvironment. No one has examined the capacity of other BM microenvironmental cells (eg, osteoblasts, BM microvascular endothelial cells, macrophages) to support or modulate B-cell development. One technical problem is the difficulty in purifying and establishing long-term cultures of human BM stromal cell components. SV40 large t antigen[127] or human papilloma virus E6/E7 genes[128] are capable of immortalizing human BM stromal cells. These stromal cell "lines" have been used to study hematopoiesis (eg, Li et al[129]), but no reports have described their capacity to support human B-cell development. Another possible strategy for long-term maintenance of BM stromal cells would be overexpression of the catalytic subunit of telomerase, which has been shown to exceed the life span of human fibroblasts by up to 20 doublings.[130] Development of stable long-term BM stromal cell cultures would facilitate the isolation of potentially novel genes that encode survival/growth factors regulating human B-cell development using, for example, the cloning/screening strategy of Oritani and Kincade.[90] A more detailed analysis of the NOD-SCID mouse might focus on whether fetal liver or BM stromal cells are comparable to human BM stromal cells in supporting human B-cell development. It is conceivable that a highly conserved murine cytokine is as effective as its human homologue, in which case murine stromal cell complementary DNA (cDNA) libraries could be screened for binding to human B-lineage cells.[90]

B-lineage immunodeficiencies

Dramatic progress has recently been made in identifying the genetic defects in many congenital human immunodeficiency diseases.[131] These diseases are largely classified on the basis of which cellular component or function of the immune response is defective.[132] By the grace of good hindsight, it is not surprising that immunodeficiency diseases that primarily affect B-cell development or B-cell function involve genes encoding protein components of the pre-BCR, BCR, or signaling pathways activated following cross-linking these receptors.[133] The degree to which B-cell development or function is altered in these patients shows many similarities and some differences compared with the phenotype observed in gene-targeted mice.

X-linked agammaglobulinemia (XLA)

XLA is the prototype immunodeficiency disease that specifically affects the B-lineage.[131-133] The Bruton's tyrosine kinase (*BTK*) gene encodes a cytosolic 659–amino acid protein that is mutated in the vast majority of boys diagnosed with XLA.[134,135] *BTK* mutations are found in 80% to 90% of patients following a presumptive diagnosis of XLA based on early-onset hypogammaglobulinemia and few or no detectable peripheral blood B cells.[136] More than 300 mutations have been identified in the *BTK* gene,[137] and mutations have been mapped to all 6 of the functional domains.[138] The majority of XLA patients have profound hypogammaglobulinemia afflicting all immunoglobulin classes and fewer than 1% of normal numbers of peripheral blood B cells. A single study of 8 patients indicated that maturation arrest occurred at the pro-B/pre-B interface, ie, between CD19+/TdT+/cytoplasmic μHC− and CD19−/TdT−/cytoplasmic μHC+ populations.[139]

The xid mouse, a murine model of X-linked immunodeficiency disease, harbors a mutation in the *Btk* gene.[140] Mice with targeted disruptions of *Btk* have a defect in B-cell development that is identical to the xid mouse.[141-143] As discussed in detail elsewhere.[131,133,140-143] xid and *Btk* gene–targeted mice have a much milder form of B-cell immunodeficiency than XLA patients, characterized by reduced levels of only 2 immunoglobulin subclasses (IgM and IgG3) and B cell numbers reduced by only 30% to 50%. Comparison of B-cell developmental defects in mice and humans led many investigators to conclude that loss of BTK function has more severe consequences in humans. This could be explained by compensatory/redundant kinases operating in murine B-cell development or by a difference in the role of BTK in murine and human pre-BCR and/or BCR signaling pathways. A more

intriguing possibility is the potential contribution of modifying genetic factors/modifier alleles (ie, their gene products) in facilitating the traversal of pro-B to pre-B cells in XLA patients.[133,136] This may explain the variability in immunologic symptoms present in family members with identical genetic backgrounds. Despite considerable effort, no correlation has been determined between XLA genotype and the severity of clinical symptoms in XLA patients. Whether human XLA has a more severe defect in B-cell development than the murine models is still a matter of some controversy, but the unique function of BTK in B-cell development in both species is undeniable.

The role of BTK in signal transduction pathways has been extensively studied, and Rawlings has recently reviewed this subject.[138] BTK is expressed throughout the B-lineage, but expression decreases in terminally differentiated plasma cells.[144,145] BTK activation following BCR cross-linking has been studied in detail.[138] Briefly, BCR cross-linking activates PI-3 kinase, which generates limiting amounts of membrane-associated PI-3,4,5-trisphosphate. The latter recruits cytosolic BTK to the membrane by interacting with the BTK SH3 domain.[146] BTK activation then proceeds through 2 steps: transphosphorylation of Y551 within the BTK kinase domain (most likely by the *src* family kinase Lyn), followed by autophosphorylation of Y223 in the BTK SH3 domain.[147] Membrane-associated BTK then binds to an unidentified tyrosine-phosphorylated ligand,[138] which facilitates colocalization of BTK with phospholipase Cγ (PLC-γ), activation of PLC-γ, and culmination in a sustained calcium signal involving extracellular calcium influx.[148] Very recent studies suggest that the "unidentified tyrosine-phosphorylated ligand" could be the B-cell linker protein (BLNK).[149] The outcome of this complex pathway leading to sustained calcium signaling is enhanced proliferation and changes in transcription. I would emphasize that this model of BTK function has been developed with the use of BCR cross-linking as an activation stimulus. Evidence that BTK functions through the same pathway following pre-BCR activation is lacking. It is possible that once the pre-BCR becomes activated (eg, through ligand-independent tonic signals as discussed above), BTK occupies a critical point in the pre-BCR signaling pathway whose function is nonredundant. Given the complexity of BTK protein domain organization, it is remarkable that so many distinct *BTK* mutations culminate in a relatively similar block in B-cell development.

Non–X-linked agammaglobulinemia

As discussed above, 10% to 20% of B-lineage immunodeficiency patients do not harbor *BTK* mutations. Mary Ellen Conley and her colleagues have systematically screened BM DNA samples from patients lacking *BTK* mutations in an effort to identify other mutated genes that could underlie these immunodeficiency diseases. By this approach, they have identified patients with mutations in the μHC,[150] the λ5/14.1 component of the ψLC,[33] and Igα.[151] Seven patients from 3 families harbored mutations that disrupted the μHC.[150] These included 75-kb to 100-kb homozygous deletions of the D and J regions plus the μ constant region, and a homozygous base-pair substitution that removed an alternate splice site used to generate the membrane form of the μHC. Analysis of peripheral blood from 4 of 7 patients revealed no detectable B cells, and analysis of the BM from 1 of the 4 evaluable patients indicated maturation arrest at the pro-B to pre-B interface.[150] In a second report, a 5-year-old boy with severe hypogammaglobulinemia was found to have fewer than 1% of the normal number of peripheral blood B cells.[33] A detailed analysis of the mutated λ5 alleles and their encoded proteins suggested that the mutated λ5 protein underwent improper folding and was subsequently degraded.[33] Analysis of this patient's BM suggested a block at the pro-B to pre-B transition. In the most recent report, a 2-year-old girl with agammaglobulinemia was found to have a deletion of exon 3 in the gene encoding Igα.[151] This exon encodes the transmembrane domain of Igα leading to the prediction that the Igα transcript made in this patient would encode a truncated protein incapable of assembling with the pre-BCR. Analysis of this patient further revealed the complete absence of peripheral blood B cells and a block at the pro-B to pre-B transition. A non-XLA patient with a block at the pro-B cell stage and a decrease in Igα, Igβ, and V_H-Cμ transcripts may represent yet another distinct mutation in genes essential for B-cell development.[152] It is remarkable that mutations in *BTK* and genes encoding components of the pre-BCR can lead to a relatively similar block in B-cell development at the pro-B to pre-B transition. Figure 4 suggests why this may occur. Expression of the pre-BCR requires assembly of the μHC, ψLC, and Igα/Igβ subunits. Any mutation that leads to an absence of one subunit will block full assembly of the pre-BCR. The physical absence of an intact pre-BCR will result in a failure of the pre-BI compartment (Figure 2) to expand. Independently of how the pre-BCR signals, the presence of the Igα/Igβ heterodimer predicts that pre-BCR and BCR signaling pathways will be highly conserved.[31,138] Thus, in XLA patients, pre-BCR cross-linking would be normal at least to the point where BTK is translocated to the membrane, but BTK-dependent events leading to a sustained increase in Ca^{++} flux would be greatly decreased or absent (Figure 4).

B-lineage ALL

In approximately 75% of pediatric patients with newly diagnosed ALL, the disease is classified as B-lineage in origin on the basis of immunoglobulin gene rearrangements and expression of cell

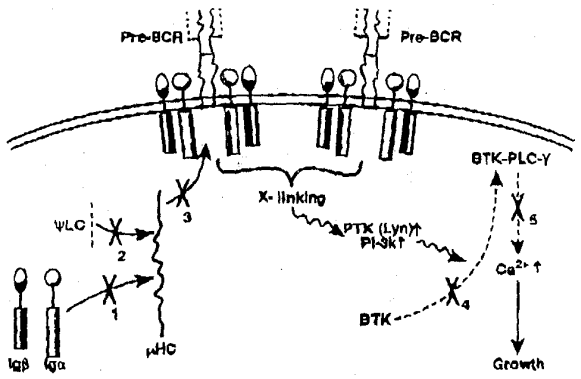

Figure 4. Components of the pre-BCR and pre-BCR signaling pathways disrupted in B-lineage immunodeficiencies. The left side shows the assembly of the structural components of the pre-BCR: μHC, ψLC, and the Igα/Igβ heterodimer. For simplicity, only the formation of a Fab is shown. As discussed in the text, the mechanism of pre-BCR cross-linking is unknown. By whatever mechanism, pre-BCR cross-linking activates protein tyrosine kinases (PTKs) such as Lyn, followed by a complex series of events (see Benschop et al[31] and Rawlings[138] for detailed reviews) culminating in initial activation of PLC-γ. Concomitant activation of *PI-3 kinase* leads to production of PI (3,4,5) P3, which recruits BTK to the membrane where it is phosphorylated by Lyn. BTK then phosphorylates PLC-γ, leading to sustained Ca^{++} flux and enhancement of growth. The assembly (solid arrows) or signaling pathways (dashed arrows) disrupted in antibody-deficiency diseases are shown by an X and a number (1 indicates mutation in Igα that would impair pre-BCR assembly; 2 indicates mutation in ψLC that would impair pre-BCR assembly; 3 indicates mutation in μHC that would impair pre-BCR assembly; 4 indicates *BTK* mutation in pleckstrin homology domain that would compromise binding to PI (3,4,5) P3; and 5 indicates *BTK* mutation in catalytic site that would compromise tyrosine phosphorylation of PCL-γ).

surface markers.[153-155] The karyotypic and molecular genetic abnormalities in B-lineage ALL have been extensively characterized,[153-155] and chromosomal translocations giving rise to distinct fusion genes including *TEL-AML1, MLL-AF4* (or MLL rearrangements with other genes), and *E2A-PBX* are present in more than 30% of newly diagnosed pediatric B-lineage ALL.[153] However, the totality of molecular genetic abnormalities in B-lineage ALL is much greater than these landmark translocations. Despite this impressive progress, there is still a deficiency in our understanding of how these many genetic abnormalities ultimately subvert normal B-cell precursor developmental programs. Related questions are how these genetic abnormalities tip the survival scale to apoptotic resistance and whether external cues (ie, cytokines) play any role in regulating the survival/growth of B-lineage ALL in vivo.

The universal common denominator of pediatric B-lineage ALL is a BM origin of the disease. However, as discussed by Greaves,[156] infant and pediatric ALLs are biologically and clinically distinct diseases. For purposes of this discussion, we will consider both of them as "B-lineage," even though infant ALL with MLL-AF4 translocations have characteristics of bi-phenotypic B-lineage/myeloid cells.[156] Manifestation of molecular genetic abnormalities shifts the B-cell precursor developmental program from (1) a process governed by functional immunoglobulin gene rearrangements and appropriate homeostatic response to positive and negative growth regulators to (2) a transformed clone more resistant to apoptosis and (generally) incapable of undergoing differentiation. The apoptotic death of normal B-cell precursors probably resembles death by neglect,[157] ie, an apoptotic fate that follows decreased availability or absence of a continuous survival signal. Neglect might reflect primarily the fate of a cell that cannot express the pre-BCR (eg, a cell with 2 nonfunctional μHC rearrangements) and hence does not receive a tonic (survival) signal that follows pre-BCR expression. Normal murine and human B-cell precursors express antiapoptotic bcl-2 family members such as bcl-2 and bcl-x,[27,158-160] but are nonetheless very sensitive to apoptotic stimuli.[158,159] Many laboratories have studied B-lineage ALL for expression of bcl-2 family members in an attempt to determine whether expression can be correlated with the clinical or biological characteristics of the disease.[161-166] The results of these studies are quite variable, and no simple conclusion can be drawn regarding bcl-2 family member expression and clinical outcome. Subcellular distribution (particularly in mitochondrial membranes) and homodimerization/heterodimerization characteristics of bcl-2 family members are crucial in determining apoptotic sensitivity in many eukaryotic cells.[167-169] It is therefore interesting that a recent study suggested that mitochondrial levels of bcl-2 may portend the sensitivity of leukemic cells to apoptosis.[164]

A fascinating relationship between a molecular genetic abnormality and apoptotic resistance in B-lineage ALL is the E2A-HLF translocation. The *E2A-HLF* fusion gene occurs as a consequence of the t(17;19)(q23;p13) in some cases of pro-B ALL.[170,171] The E2A-HLF fusion protein contains the transactivation domains of the transcription factor E2A tethered to the basic leucine zipper DNA–binding domain of the transcription factor HLF. Inaba and colleagues demonstrated that a dominant-negative form of E2A-HLF induced apoptosis in a human pro-B ALL cell line harboring the E2A-HLF translocation, and transfection of a murine IL-3–dependent pro-B cell line with *E2A-HLF* reversed apoptosis that normally occurred following IL-3 withdrawal.[172] These data strongly suggest that E2A-HLF functions by blocking an early step in an apoptotic pathway.[172] Reasoning that IL-3 mediates cell survival by activation of 1 or more transcription factors whose activity can be substituted by E2A-HLF, the same group went on to show that nuclear factor regulated by IL-3 (*NFIL3*) is a target gene of E2A-HLF.[173] Enforced expression of NFIL3 promoted the IL-3–independent survival of pro-B cells.[173] Two recent studies used representational difference analysis to identify additional genes regulated by E2A-HLF.[174,175] One study identified Annexin VIII and a novel cDNA designated SRPUL, but neither protein prevented apoptosis in murine pro-B cells deprived of IL-3.[174] In the second study, E2A-HLF was shown to up-regulate a zinc-finger transcription factor designated SLUG.[175] Importantly, SLUG was nearly as effective as bcl-2 or bcl-x in preventing apoptosis in IL-3–deprived pro-B cells.[175] Murine models of E2A-HLF mediated oncogenesis have been developed.[176,177] In both studies, E2A-HLF transgenic mice exhibited thymic hypoplasia and subsequent development of thymic lymphomas. Although a block in splenic B-cell maturation was noted in one of the studies,[177] leukemias involving B-lineage progenitors were rare.

A second fusion gene containing E2A that is grudgingly giving up its function is E2A-PBX1. The *E2A-PBX1* fusion gene was originally identified in pre-B ALL blasts harboring the t(1;19)(q23;p13) cytogenetic abnormality by two groups.[178,179] The E2A-PBX1 fusion protein contains the N-terminal domain of E2A fused to the homeodomain of PBX1. This genetic abnormality is specific for pre-B ALL expressing cytoplasmic μHC and is present in approximately 25% of newly diagnosed pre-B ALL (reviewed in Hunger[180]).Transgenic mice expressing the *E2A-PBX1* fusion gene develop thymic lymphomas and myeloid leukemias, but not B-lineage malignancies.[181,182] Surprisingly, BM B-lineage cells are reduced to 20% of normal values in E2A-PBX1 transgenic mice, suggesting that the fusion protein increases the sensitivity of these cells to apoptosis.[181] Representational difference analysis was used to isolate a novel *WNT* gene, designated WNT-16, as an activating target of E2A-PBX1.[184] WNT-16 is a member of the vertebrate WNT family, which includes more than 20 genes encoding cysteine-rich secreted proteins that mediate cell-cell interactions.[183] WNT-16 mRNA is expressed in E2A-PBX1$^+$ pre-B ALL but not a variety of E2A-PBX1$^-$ B-lineage malignancies.[184] Furthermore, *Frizzled* genes that encode receptors for WNT family members are expressed in B-lineage ALL, including those expressing E2A-PBX1. These data implicate WNT-16 as one component in a survival/growth pathway that is operative in pre-B ALL harboring the *E2A-PBX1* fusion gene.[184]

The earliest stages of clonal expansion in B-lineage ALL (ie, the subclinical phase of the disease wherein the progeny of a single clone begin to expand) may be characterized by a dependency on BM stromal cells for survival and growth. This would be a stage in the natural history of the disease in which the BM microenvironment is completely intact and lymphohematopoiesis is unperturbed. How long this BM stromal cell dependency might be retained is unknown. Acquisition of sequential genetic changes may portend the emergence of a dominant subclone with a decreased, or complete absence of, a requirement for BM stromal cell–derived survival/growth factors. By the time a patient is diagnosed with B-lineage ALL and the marrow is filled with leukemic blasts, a physical displacement of normal lymphohematopoiesis and BM architecture will have occurred. Indeed, physical disruption/displacement of the BM stromal cell microenvironment is more frequently seen in ALL than in AML or CML.[185] It is likely that a dominant B-lineage ALL subclone would be BM stromal cell–independent at this stage.

In addition to the subversion of apoptotic programs by genetic

changes in B-lineage ALL, are there external cues that could regulate survival/growth? Several laboratories have examined the effect of recombinant cytokines on the growth of B-lineage ALL using short-term in vitro assays. Sporadic responsiveness to IL-3, IL-7, and flt3-ligand was observed.[186-195] However, no single cytokine has been demonstrated to exert a consistent proliferative effect on a significant percentage of cases. Furthermore, the response to these cytokines (ie, the degree of proliferation) is generally weak. A potentially more rational approach to identifying growth factors is to assume that BM stromal cells produce the collective array of survival/growth factors essential for clonal expansion of B-lineage ALL. This assumes that adhesive interactions exist to bring the leukemic clone into apposition with BM stromal cell surfaces and the surrounding extracellular matrix. Similarly to their normal counterparts,[107-109] B-lineage ALL cells generally adhere to BM stromal cells through VLA-4/VCAM-1 interactions.[109,196-198] Using a fluorescent bead adhesion assay that facilitated flow cytometric analysis of integrin expression/function, Geijtenbeek and colleagues reported that leukemic cells from 17 of 20 B-lineage ALL BM specimens exhibited defects in expression or activation of LFA-1 and VLA-4.[199] The biological significance of their results is uncertain. On the one hand, weaker or reduced adhesion of leukemic cells to BM stromal cells could lead to more rapid egress into the peripheral blood. On the other hand, interaction of leukemic cells with BM stromal cells generally inhibits apoptosis (see below), indicating that adherence could play an important role in the survival/growth of B-lineage ALL.

Adherence of B-lineage ALL cells to BM stromal cells could be followed by a more complex, energy-dependent interaction, characterized by migration of the leukemic cells underneath BM stromal cells. Interestingly, migration (at least in vitro) is VCAM-1–independent.[196,198] The biological significance of in vitro migration is unclear, but may reflect a chemotactic response by the leukemic cells to BM stromal cells. The CXCR4 chemokine receptor and its SDF-1 ligand may be involved in leukemic cell migration since at least some B-lineage ALLs undergo chemotaxis in response to SDF-1.[102,103] SDF-1 may also promote the survival of B-lineage ALL.[200]

Campana's laboratory has extensively examined the capacity of nontransformed human BM stromal cells to inhibit the apoptotic fate of freshly isolated B-lineage ALL. They initially showed that allogeneic BM stromal cells support survival or inhibit apoptosis of the majority of B-lineage ALLs tested, although survival of a minority of B-lineage ALL was unaffected.[201] They went on to demonstrate that direct contact with BM stromal cells was necessary for optimal survival of normal B-cell precursors and some (but not all) B-lineage ALLs.[202] Heterogeneity in BM stromal cell contact requirements for B-lineage ALL survival/growth was also reported by other investigators.[203,204] The survival of B-lineage ALL on allogeneic BM stromal cells also correlates with prognosis. The probability of 4-year event-free survival was greater among patients whose leukemic cells exhibited reduced survival on BM stromal cells, compared with patients whose leukemic cells exhibited elevated survival on BM stromal cells.[205] A very recent report from Campana's group provided strong evidence that hyperdiploidy (51 to 65 chromosomes) in B-lineage ALL showed a significant correlation with reduced capacity of the leukemic cells to survive on BM stromal cells.[206] This is a strong endorsement for the utility of this biological assay in predicting clinical outcome and likely reflecting the in vivo apoptotic sensitivity of this subcategory of B-lineage ALL.

Many B-lineage ALL cell lines have been established, but the vast majority (if not all) require only supplementation of tissue culture medium with fetal bovine serum for optimal growth.[207] My own laboratory has established a panel of human B-lineage ALL cell lines that retain a dependency on human BM stromal cells for long-term survival and growth. Using the same human BM stromal cell culture employed for studies of normal B-cell precursors,[26,81] we established a cell line designated BLIN-2 (B-lineage 2).[208] BLIN-2 cells express the pre-BCR, have a dic(9;20) chromosomal abnormality and a bi-allelic deletion of the $p16^{INK4a}$ and $p14^{ARF}$ genes. BLIN-2 has an absolute dependence on human BM stromal cells for survival and growth, and direct contact is necessary for optimal growth. Removal of BLIN-2 from BM stromal cells results in membrane blebbing and apoptotic body formation in 72 hours. Using a variety of assays to characterize apoptotic fate, we have recently shown that BLIN-2 cell death has caspase-dependent and caspase-independent features.[209] Although the identity of the BM stromal cell molecules that are essential for growth of BLIN-2 are unknown, heparan sulfate proteoglycans may play at least a partial role.[210] We have also produced 2 additional cell lines, designated BLIN-3 and BLIN-4, that have overlapping but unique growth factor requirements compared with BLIN-2.[210] BLIN-3 requires human BM stromal cells supplemented with exogenous IL-7 for optimal growth, survives but does not proliferate in the presence of BM stromal cells alone, and undergoes apoptosis in the absence of BM stromal cells.[210] BLIN-4 grows on BM stromal cells and undergoes apoptosis in their absence. However, growth of BLIN-4 can be supported by a cooperative stimulus of exogenous IL-7 plus flt3-ligand in the absence of BM stromal cells.[210] The BLIN cell lines represent a composite of growth-factor requirements that may mirror the physiologic dependency of normal and leukemic B-cell precursors on the BM microenvironment.

Conclusion

The general blueprint for mammalian B-cell development has been determined, and the investigative fine-tuning has begun. A number of questions regarding human B-cell development remain unanswered. For example, how does a B-lineage cell develop from a multilineage progenitor (eg, a CLP in Figure 1), and how is B-lineage commitment defined in molecular terms? Transcription factors are obviously the key. One of the great accomplishments in hematology during the nineties was the isolation and characterization of transcription factors that regulate the development of murine lymphohematopoietic lineages (for recent reviews, see Glimcher et al[211] and Engel et al[212]). A stunning recent discovery directly implicated the paired box transcription factor PAX5 in murine B-lineage commitment.[213,214] The major message from these 2 studies is that PAX5-deficient murine pro-B cells (ie, B-lineage cells that have undergone DJ_H but not VDJ_H rearrangements) harbor the capacity to differentiate into a constellation of other lineages—including macrophages, osteoclasts, dendritic cells, granulocytes, NK cells, and thymocytes.[213,214] This surprising result was used to propose that PAX5 plays an essential role in fostering B-lineage commitment by suppressing the expression of genes that (directly or indirectly) promote development of non-B lineage cells. It is reasonable to assume that human B-lineage commitment and development are governed by similar transcription factors, but is there experimental evidence? Answers may be forthcoming. Jaleco and colleagues have very recently described a strategy that represents the first success in elucidating the role of transcription factors in human B-cell development. They constructed a retroviral vector encoding green fluorescent protein

(GFP) and the dominant negative helix loop helix protein Id3.[215] Human fetal liver HSCs were then infected with this vector and plated on murine or human stromal cells, and GFP expression was used to trace the effect of overexpression of Id3 on B-cell development. The results indicated that Id3 overexpression blocked B-cell development at a stage prior to expression of the IL-7 receptor.

Another issue that requires resolution is the identity of the molecule (or molecules) produced in the BM microenvironment that are essential for the survival/proliferation of human B-cell precursors. I propose that stromal cell–derived molecules potentially bound to HSPGs (Figure 3) are reasonable candidates. Time will tell if these molecules turn out to be previously cloned cytokines/chemokines. These putative cytokines/chemokines could also play an important role in the survival/proliferation of at least some B-lineage ALL. The intracellular signaling pathways that affect survival/proliferation in normal and leukemic B-lineage cells are not completely understood. Very recent reports reveal a critical role for the linker protein BLNK in human[216] and murine[217,218] B-cell development, although the absence of BLNK function may result in a more severe phenotype in humans than mice. Thus, additional efforts will lead to the discovery of new components, or novel functions for known components, in signaling pathways essential for the proliferation and differentiation of B-cell precursors. The identity of the essential survival/proliferation factor is linked to a related question: what is the mechanism of cell death that ensues in a B-cell precursor that does not receive a survival/proliferation signal (eg, in a pre-BI cell that fails to express the pre-BCR)? Which caspase pathways are involved? Are these pathways subverted in B-lineage ALL and accentuated in XLA?

Finally, "genome prospecting,"[219] using DNA microarray technology with all its analytical power and bio-informatic challenges, has burst onto the scene. Golub and colleagues used DNA microarrays to evaluate gene expression in human acute leukemias, included B-lineage ALL.[220] Their results indicate that microarray-based quantitation of gene expression (1) confirms well-known leukemia classifications, (2) provides a new tool for diagnosis, and (3) generates a staggering amount of new information of unknown significance (ie, quantitative expression of approximately 6800 human genes). Once this technology is applied to normal B-cell precursors, we will witness the beginning of a complete fingerprint of comparative gene expression. This database will provide an investigative substrate for the next millenium, taking us deeper into regulation of cell fate/function in normal and abnormal human B-cell development.

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B cell development and activation defects resulting in *xid*-like immunodeficiency in BLNK/SLP-65-deficient mice

Shengli Xu, Joy En-Lin Tan, Esther Poh-Ying Wong, Arunkumar Manickam, Sathivel Ponniah and **Kong-Peng Lam**

Institute of Molecular and Cell Biology, National University of Singapore, 30 Medical Drive, Singapore 117609, Republic of Singapore

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Abstract

Engagement of the B cell receptor (BCR) leads to the activation of tyrosine kinases and other signaling molecules that ultimately determine the type and magnitude of the B lymphocyte's cellular response. The adaptor protein BLNK/SLP-65 plays a pivotal role in BCR signal transduction by coupling Syk activation to downstream elements such as Grb2, phospholipase C-γ, Vav and Nck. We have generated $BLNK^{-/-}$ mice to determine the physiological role of this protein in B cell development and activation. $BLNK^{-/-}$ mice exhibit an incomplete block in B cell development with a severe inhibition of pro-B to pre-B cell differentiation. $BLNK^{-/-}$ $sIgM^+$ cells can develop, seed the peripheral lymphoid tissues and accumulate in numbers overtime. However, these mutant B cells failed to mature and are non-responsive to BCR cross-linking in terms of proliferation and up-regulation of activation markers such as CD69 and CD86 (B7-2). In addition, the $CD5^+$ subset of B cells is absent. The immune response to T cell-independent antigen but not T cell-dependent antigen is also impaired. Overall, the phenotype of $BLNK^{-/-}$ mice bears a striking resemblance to that of *xid* mice which is the murine model of human *XLA* that has a mutation in Bruton's tyrosine kinase. This raises the interesting possibility that mutation in BLNK/SLP-65 may be responsible for certain human immunodeficiencies.

Introduction

The pre-B cell receptor (pre-BCR) and the BCR play pivotal roles in the development of B lymphocytes. The pre-BCR comprising the Ig heavy chain and surrogate light chains, and the BCR that is composed of the surface Ig, are complexed to the signal transducing subunits Igα and Igβ (1). Studies with the μMT mouse that has a targeted deletion of the transmembrane exon of the Ig heavy chain (2) or the λ5T mouse that lacks surrogate light chain (3) indicated that the inability of these mutant mice to express a pre-BCR can lead to the arrest of B cell development at a very early stage. In addition, mice with a compromised BCR resulting from the truncation of the cytoplasmic tail of Igα (4) do not accumulate mature B cells in the periphery. Finally, the induced ablation of BCR on mature peripheral B cells leads to their rapid cell death (5). Taken together, these studies implied that signals from the pre-BCR and BCR are required for the progression of B lymphopoiesis and the maintenance of B cell survival.

Signal transduction events have been studied extensively in B lymphocytes. Engagement of the BCR activates cytoplasmic protein tyrosine kinases such as Syk, Lyn, Blk and Bruton's tyrosine kinase (Btk) (6), and can lead to a multitude of cellular responses, such as proliferation, activation, differentiation or cell death. The current challenge in the field of B cell signaling is to identify specific signaling pathways that associate with a particular cellular response. Recently, it has been demonstrated that adaptor proteins play a major role in interfacing tyrosine kinase activation by lymphocyte antigen receptors with selective downstream signaling molecules. One such adaptor molecule termed BLNK (7), SLP-65 (8) or BASH (9) has been identified in B cells and is specifically involved in BCR signaling. BLNK can associate with Btk (10) and also couple Syk activation to Grb2, phospholipase C (PLC)-γ, Vav and Nck (7), and is intimately associated with intracellular Ca^{2+} mobilization which is essential for cell activation (11).

Correspondence to: K.-P. Lam

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BLNK contains a C-terminus SH2 domain, several SH3 domains and a series of YXXP motifs in the N-terminus (7–9). It bears striking homology to another adaptor protein SLP-76 that is expressed in T cells (12) and intimately involved in TCR signaling. In general, the order of the signaling events from the TCR and BCR is quite similar, with the engagement of the antigen receptors triggering the activation of similar classes of intracellular cytoplasmic kinases. In analogy to BLNK in B cells, SLP-76-coupled TCR induced ZAP-70 (the equivalent of Syk) activation to Ca^{2+} mobilization in T cells (13). In addition, SLP-76 is essential for T cell development as its inactivation in the mouse germline leads to a profound block in thymocyte maturation at a very early stage (14,15). Thus, given the central role of BLNK in BCR signaling and its similarity to SLP-76 in T cells, we have inactivated BLNK in the mouse to study its physiological role in B cell development and activation.

Methods

Generation of BLNK/SLP-65-deficient mice

The cDNA for BLNK/SLP-65 was obtained by RT-PCR of RNA isolated from mouse spleens using primers 5′-AGTGGCTTGAGTTCTTGAGGC-3′ and 5′-AGAAAAGCTCGTGTGAACGCC-3′, and used to screen a mouse 129 genomic DNA library. Restriction enzyme digestion, Southern blotting and DNA sequencing were used to map a phage clone containing some 5′ exons of BLNK. Subsequently, a targeting vector was constructed to replace the exon containing the starting ATG and a further 4.5 kb of DNA upstream with a *neo* gene. A 5 kb *Bam*HI–*Cla*I fragment 5′ and a 2 kb *Nhe*I–*Bam*HI fragment 3′ of the deleted exon were used as the long arm and short arm of homology respectively. To inactivate BLNK in the germline, 10^7 E14.1 embryonic stem (ES) cells were electroporated with 10 μg of *Not*-linearized targeting vector and selected with 300 μg/ml G418 (Gibco, Hong Kong, ROC) and 2 μM gancyclovir. Double-drug-resistant ES cell clones were screened by Southern blotting for homologous recombinants using probe A as shown in Fig. 1. The frequency of targeting was 1:100. Two ES cell clones with the correct configuration of the targeted locus were injected into C57BL/6 blastocysts to generate chimeric mice for germline transmission of the mutant allele.

Antibodies

The following mAb used in the flow cytometry analyses were purchased from PharMingen (San Diego, CA): anti-B220 (RA3-6B2); anti-IgM (331.12), anti-IgD (1.3-5), anti-CD43 (S7), anti-CD5 (53-7), anti-CD11b (M1/70), anti-CD23, anti-CD69, anti-CD86 (B7-2), anti-μ^a(DS-1) and anti-μ^b (AF6-78.25). The goat anti-mouse IgM F(ab)′$_2$ fragment used in the *in vitro* stimulation assays was obtained from Chemicon (Temecula, CA).

FACS analyses

Tissues and cell preparations for flow cytometric analyses were prepared as previously described (16). In brief, single-cell suspensions were obtained from spleen and lymph nodes by dissociation of these tissues with a plastic mesh and a rubber-stopper from a 5 ml syringe. Peritoneal cavity and bone marrow cells were obtained by injecting staining medium (PBS containing 3% FCS and 0.1% NaN_3) into the peritoneal cavity and femur and tibia respectively using a 10 and 1 ml syringe with a 26-gauge needle. All cells were treated with red blood cell lysing solution (0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to eliminate erythrocytes. For FACS analyses, cells were stained with optimal amounts of FITC-, phycoerythrin (PE)- and biotin-conjugated mAb for 10 min on ice, and washed 3 times with staining medium. Biotin-conjugated mAb were revealed with streptavidin–CyChrome. Flow cytometry analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA).

In vitro stimulation and proliferation assays

Splenic B cells were obtained from wild-type and mutant mice by negative selection using MACS (Miltenyi Biotech) with anti-CD43 mAb-coupled magnetic beads that bind T cells and macrophages. The purity of B cells obtained was >90% as assessed by anti-B220 and anti-IgM mAb staining in FACS analysis. For the *in vitro* stimulation assay, 10^6 purified B cells were seeded into 48-well tissue culture plate and incubated with 10 μg/ml goat anti-mouse IgM F(ab)′$_2$ fragment overnight in RPMI medium supplemented with 10% FCS. Cells were harvested and stained for the expression of activation markers. A colorimetric MTT assay (Roche, Singapore) was used according to the manufacturer's instructions to measure cell proliferation *in vitro*. Briefly, 5×10^5 purified B cells were stimulated with varying concentrations of goat anti-mouse IgM F(ab)′$_2$ fragment in a 96-well tissue culture plate. After 48 h, the cells were incubated with the MTT labeling reagent for a further 4 h followed by the addition of solubilization solution overnight. Cell proliferation was quantified using an ELISA reader at 570 nm wavelength.

Immunizations of BLNK/SLP-65-deficient mice

The ability of BLNK$^{-/-}$ mice to mount a humoral immune response was assessed by immunizing the animals with the hapten 4-hydroxy-3-nitrophenyl acetyl (NP). Wild-type and mutant mice were immunized i.p. with 10 μg NP_{25}-Ficoll in PBS to examine their immune responses to a T cell-independent antigen. For the immune response to a T cell-dependent antigen, mice were immunized i.p. with 100 μg alum-precipitated NP_{17}-chicken globulin (CG). Sera were obtained from mice at day 0 and 8 of immunizations to detect the presence of NP-specific antibodies in an ELISA. To detect NP-specific antibodies, the ELISA plates were coated with 50 μl of 5 μg/ml NP-BSA and blocked with 3% BSA. Pre-immune and immune sera were added at various dilutions to the wells of the ELISA plates. Specific antibodies of class IgM and IgG3 were quantified for the T-independent, and IgM and IgG1 for the T-dependent immune responses respectively.

Results

Generation of BLNK/SLP-65-deficient mice

BLNK/SLP-65-deficient mice were generated by deleting the exon containing the starting codon ATG and a further 4.5 kb of DNA upstream in mouse ES cells (Fig. 1). The deletion of

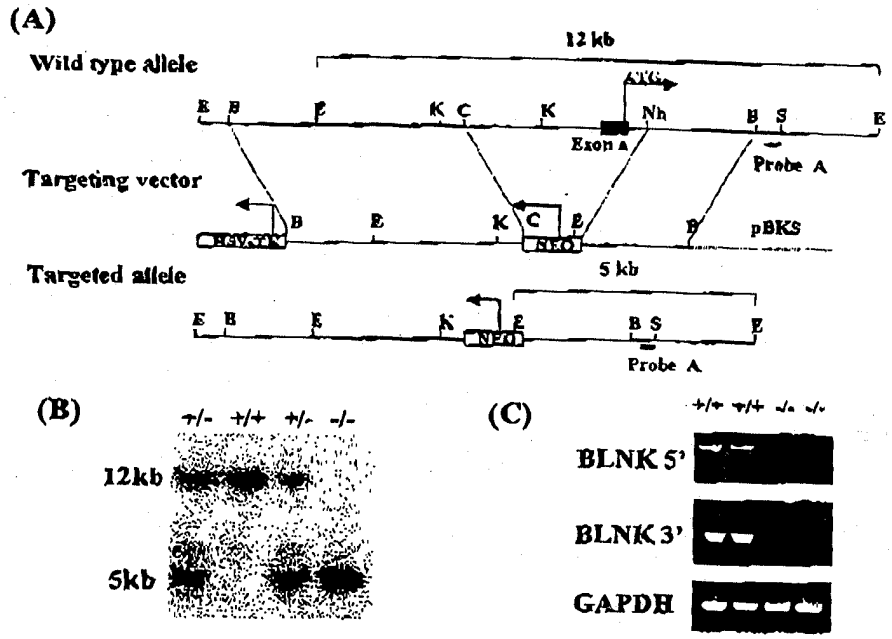

Fig. 1. (A) Inactivation of BLNK/SLP-65 in the mouse germline. Partial restriction endonuclease map of the wild-type allele, the targeting vector and the inactivated allele of BLNK/SLP-65 are shown (*Bam*HI, B; *Cla*I, C; *Eco*RI, E; *Kpn*I, K; *Nhe*I, Nh; *Sac*I, S; plasmid Bluescript, pBKS). The black box indicates the exon containing the starting ATG that is being replaced by the *neo* gene. *Eco*RI digestion of genomic DNA will yield fragments of 12 and 5 kb, as revealed by probe A for the wild-type and targeted alleles respectively. (B) Southern blot analysis of *Eco*RI-digested tail DNA obtained from wild-type, BLNK$^{+/-}$ and BLNK$^{-/-}$ mice. (C) RT-PCR of bone marrow samples obtained from wild-type and BLNK$^{-/-}$ mice. The 5′ and 3′ RT-PCR identified the regions corresponding to bp 38–396 and 999-2013 of the SLP-65 cDNA respectively. The RT-PCR for the housekeeping gene GADPH is included as controls.

the exon containing the ATG was verified by Southern blotting (Fig. 1) and by DNA sequencing (data not shown). Two targeted ES cells were injected into mouse blastocysts to generate chimera that were subsequently bred to produce mice carrying a germline mutation of BLNK/SLP-65. Homozygous mutant mice obtained were designated BLNK$^{-/-}$. The gene targeting strategy and the derivation of homozygous mice are depicted in Fig. 1(A and B respectively).

To ensure the inactivation of BLNK, RT-PCR was performed on bone marrow and spleen samples with primers that correspond to the 5′ and 3′ portions of the BLNK cDNA. As shown in Fig. 1(C), no BLNK message was detected in the samples obtained from mutant mice compared to those from wild-type control. Thus, the BLNK locl have been disrupted.

Initial flow cytometric analyses using cell surface markers suggest that there were no detectable defects in the development of macrophages, T, NK or dendritic cells (data not shown), consistent with the fact that BLNK is not shown to be expressed in these cell types (7–9). The major defect of BLNK$^{-/-}$ mice lies in the development and function of the B lineage cells, and that is the focus of our subsequent analyses.

Severe but incomplete block in B cell development in the bone marrow of BLNK/SLP-65-deficient mice

To determine the effect of BLNK/SLP-65 inactivation in early B cell development, we analyzed bone marrow cells of mutant and wild-type mice by flow cytometry. As shown in Fig. 2 (upper panel) and Table 1, immature B220^{+}IgM^{+} B cells can be found in 8-week-old mutant mice although they were reduced considerably by ~3-fold compared to wild-type control. In addition, the population of re-circulating B220^{high}IgM^{low} cells was also largely diminished by 2- to 4-fold. To gain better insight into the specific B cell stage in which the BLNK/SLP-65 mutation manifests its effect, B220^{+}IgM^{-} cells in the bone marrow were further stained with anti-CD43 mAb to resolve pro-B and pre-B cells (17). As can be seen in Fig. 2 (lower panel), BLNK/SLP-65-deficient mice lack a population of B220^{+}CD43^{-} pre-B cells that was reduced by 5-fold compared to wild-type animals. In addition, there was a 2-fold accumulation of B220^{+}CD43^{+} pro-B cells in the bone marrow of these mutant mice. The increase in the proportion of pro-B cells in BLNK$^{-/-}$ mice reflects an increase in the number of these cells as compared to wild-type mice (Table 1). Thus, the inactivation of BLNK/SLP-65 results in a severe inhibition of pro-B to pre-B cell transition. However, the block in B cell development is incomplete as a small pool of IgM^{+} B cells is generated.

BLNK is not required for the maintenance of peripheral B cells

Flow cytometric analyses of spleen and lymph nodes of BLNK$^{-/-}$ mice indicate that B220^{+} IgM^{+} B cells can be found in the peripheral lymphoid tissues although they are reduced in numbers considerably (Figs 3 and 4). This suggests that developing BLNK$^{-/-}$ B cells can exit the bone marrow environment and seed the peripheral lymphoid organs.

Expression of a BCR is required for the persistence of B

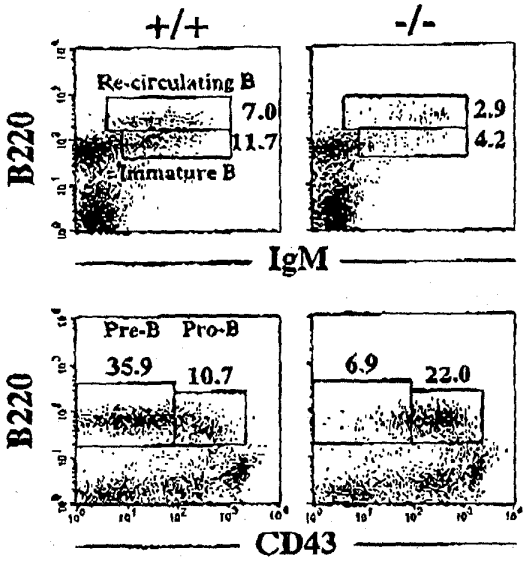

Fig. 2. Flow cytometry analyses of bone marrow cells from wild-type and BLNK$^{-/-}$ mice. Cells were obtained from the femur and tibia of 8-week-old mice, and stained with FITC–anti-IgM, PE–anti-B220 and biotin–anti-CD43 (S7) mAb. The latter was revealed by streptavidin–CyChrome. The upper panel depicts the B220 versus IgM staining of bone marrow cells, whereas the lower panel depicts the B220 versus CD43 profiles of IgM^{-} bone marrow cells. B cells at different stages of development are indicated. Numbers indicate percentage of cells in the lymphocyte gate. Representative of >10 analyses.

Table 1. Bone marrow B cell populations ($\times 10^6$) in wild-type and BLNK$^{-/-}$ mice

Genotype	*n*	Pro-B	Pre-B	Immature B	Re-circulating B
+/+	6	0.91 ± 0.24	2.17 ± 0.16	1.43 ± 0.20	0.81 ± 0.27
-/-	4	2.20 ± 0.50	0.56 ± 0.29	0.57 ± 0.20	0.21 ± 0.09

Cells were obtained from one femur and tibia of mice that were 6–8 weeks old.

The number of B cells at each developmental stage was estimated on the basis of total cell count and flow cytometric analyses as shown in Fig. 2.

cells in the peripheral lymphoid tissue (5). It is postulated that the BCR provides a low-level survival signal to the peripheral B lymphocytes that is distinct from the signal that is required to activate them (18). To determine whether signal transduced by BLNK is required for the maintenance of B cells in the periphery, we examine the number of B cells in the spleens of BLNK$^{-/-}$ mice of varying age. As shown in Fig. 3(A and B), the number of B cells that are found in 3-week-old BLNK$^{-/-}$ mice is drastically reduced by 30-fold compared to control mice of similar age. However, as the mice grow older, the reduction in B cell numbers compared to control mice of equivalent age decreases, such that by 6 and 12 weeks of age, BLNK mutant mice have only 7- and 2-fold fewer B cells than wild-type animals of comparable age respectively. This

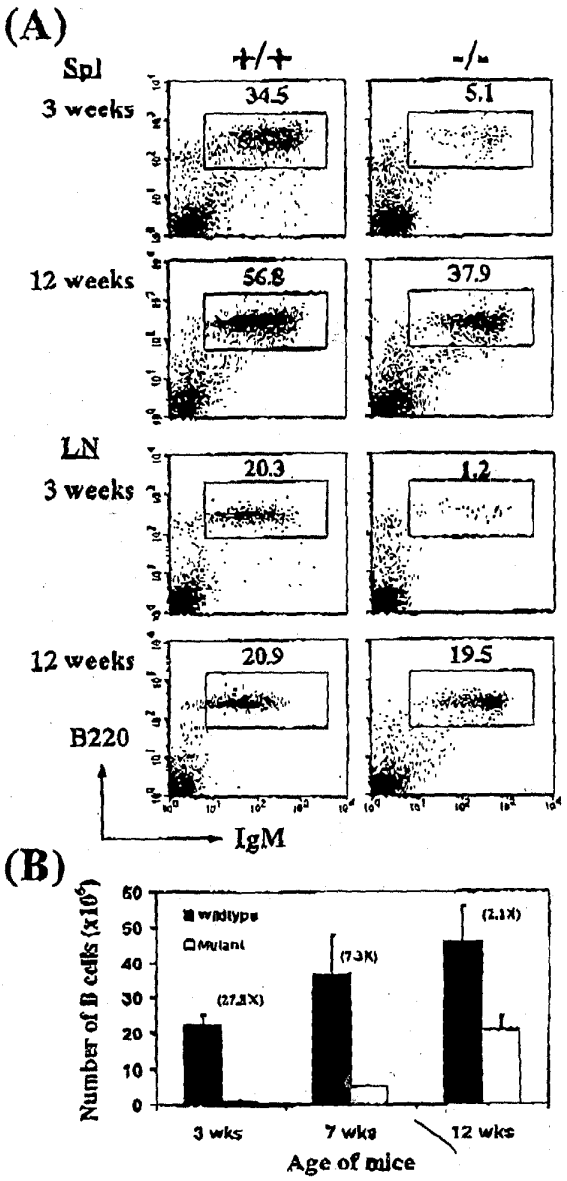

Fig. 3. Flow cytometry analysis of spleen and lymph node cells from wild-type and BLNK$^{-/-}$ mice. (A) Spleen and lymph node cells of 3- and 12-week-old wild-type and BLNK$^{-/-}$ mice were stained with FITC–anti-IgM and PE–anti-B220 mAb to resolve for the presence of B lineage cells in the periphery. Numbers indicate the percentage of cells in the lymphocyte gate. Representative of more than three analyses of mice for each age group. (B) Number of B cells found in the spleens of 3-, 7- and 12-week-old wild-type and BLNK$^{-/-}$ mice as estimated by total splenic cell count and flow cytometry analyses as shown in (A). The fold difference in the number of B cells between wild-type and mutant mice of similar age is indicated for each age group. Analyses include more than four mice for each age group.

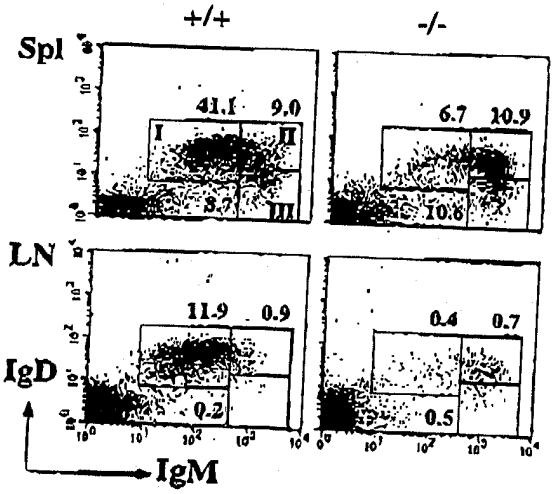

Fig. 4. Severe reduction of $IgM^{low}IgD^{high}$ Fraction I cells in the spleen and lymph node of $BLNK^{-/-}$ mice. Spleen and lymph node cells from 6-week-old wild-type and $BLNK^{-/-}$ mice were stained with FITC-anti-IgD and PE-anti-IgM mAb to depict cells form Fractions I ($IgM^{low}IgD^{high}$), II ($IgM^{high}IgD^{high}$) and III ($IgM^{high}IgD^{low}$). Numbers indicate percentage of cells in the lymphocyte gate. Representative of more than five analyses.

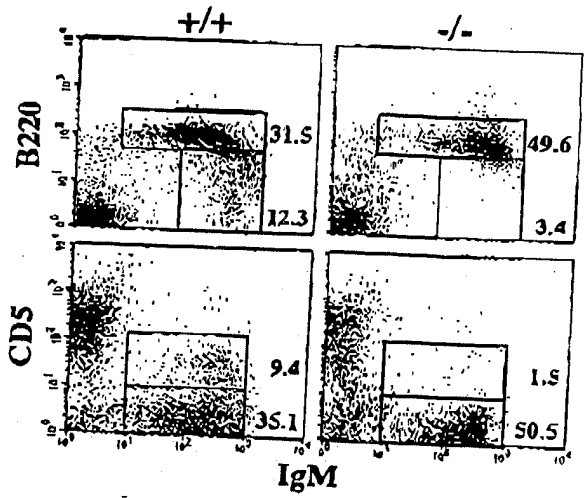

Fig. 5. Absence of $CD5^{+}IgM^{+}$ cells in the peritoneal cavity of $BLNK^{-/-}$ mice. Peritoneal cavity cells were obtained from 3-month-old wild-type and $BLNK^{-/-}$ mice and stained with FITC-anti-IgM and PE-anti-B220 (upper panel) or FITC-anti-IgM and PE-anti-CD5 (lower panel) mAb. Numbers indicate percentage of cells in the lymphocyte gate. Representative of more than five analyses.

suggests that the number of peripheral B cells in BLNK mutant mice can accumulate with age. Thus BLNK is apparently not required for the maintenance of B cells in the periphery. The accumulation of B cells in $BLNK^{-/-}$ mice with age contrasted sharply with the situation in mb-1Δc/Δc mice (4) that have a truncation of the cytoplasmic tail of Igα leading to the expression of a compromised BCR. In mb-1Δc/Δc mice the peripheral B cell pool remains diminished regardless of the age of the animals.

Severe reduction of $IgM^{low}IgD^{high}$ (Fraction I) B cells in the spleen and lymph nodes of BLNK/SLP-65-deficient mice

Peripheral B cells can be subdivided into Fractions I, II and III on the basis of differential IgM and IgD expression, and represent different stages of B cell maturation (19). Cells in Fractions III ($IgM^{high}IgD^{low}$) and II ($IgM^{high}IgD^{high}$) are the newly emigrating or transitional B cells, whereas cells in Fraction I ($IgM^{low}IgD^{high}$) are the mature B lymphocytes (20). Interestingly, as seen in Fig. 4, most of the peripheral B cells present in the spleen of an 8-week-old $BLNK^{-/-}$ mouse have an immature phenotype, and are found mainly in Fractions III and II. This is in contrast to wild-type mice in which the majority of the peripheral splenic B cells are found in Fraction I. This block in peripheral B cell maturation is even more evident in the lymph nodes of $BLNK^{-/-}$ mice compared to control animals (Fig. 4) where in the latter all cells are found in Fraction I. Thus, although $BLNK^{-/-}$ B cells can seed the peripheral lymphoid tissues and accumulate in numbers, the majority of them do not differentiate to the $IgM^{low}IgD^{high}$ mature B cell stage.

Absence of $CD5^{+}$ B cells in the peritoneal cavity of BLNK/SLP-65-deficient mice

Other than the conventional or B-2 cells found in the spleen and lymph nodes, another major subset of B cells, designated B-1 cells, exists, and these cells are found mainly in the pleural and peritoneal cavities. These cells can be distinguished from conventional B cells by their cell surface phenotype. In contrast to B-2 cells that express high levels of B220 and IgD, and moderate levels of IgM, B-1 cells express low levels of B220 and IgD, and high levels of surface IgM. In addition, they express CD5, a marker found on T cells, and do not express CD23 (21).

Flow cytometric analyses of 6-week-old $BLNK^{-/-}$ mice indicate a scarcity of B cells in the peritoneal cavity of these mice compared to wild-type control (data not shown). Since B-1 cells accumulate with age, we examine the peritoneal cavity cells of 3-month-old $BLNK^{-/-}$ mice. Detail analyses revealed that most of the cells present in $BLNK^{-/-}$ mice are conventional or B-2 cells, with the noticeable absence of the $B220^{low}IgM^{high}$ and $CD5^{+}IgM^{+}$ B cells (Fig. 5). This suggests that BLNK is required for the generation of B-1 cells.

BLNK/SLP-65-deficient B Cells cannot be activated and do not proliferate in response to anti-IgM stimulation in vitro

Cross-linking of BCR activates B lymphocytes, resulting in their up-regulation of co-stimulatory and activation molecules. To determine whether $BLNK^{-/-}$ B cells are functional and responsive to external stimuli, we treated purified splenic B cells from wild-type and $BLNK^{-/-}$ mice with anti-IgM mAb *in vitro*. As shown in Fig. 6(A), anti-IgM activated wild-type B cells up-regulate their expression of CD69, an early activation marker, as well as the co-stimulatory molecule, CD86 (B7-2). In contrast, $BLNK^{-/-}$ B cells did not up-regulate either the

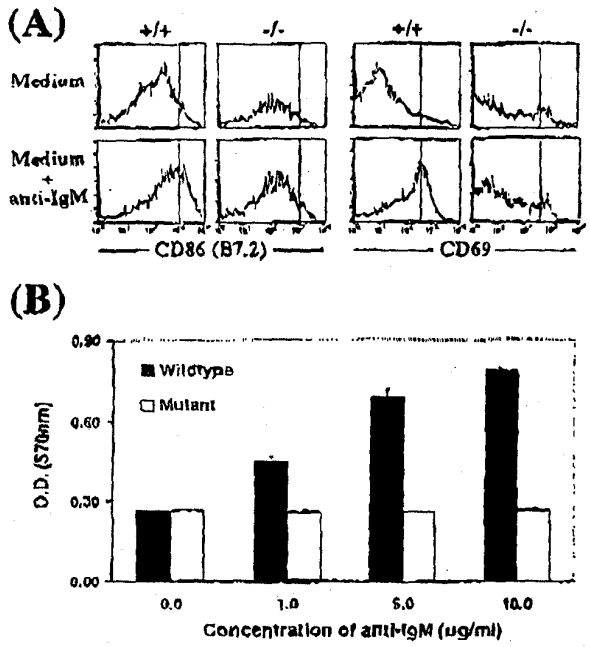

Fig. 6. BLNK$^{-/-}$ B cells cannot be activated *in vitro*. (A) BLNK$^{-/-}$ B cells do not up-regulate CD86 and CD69 in response to anti-IgM stimulation. Purified splenic B cells from wild-type and BLNK$^{-/-}$ mice were incubated in medium alone or with 10 µg/ml goat anti-mouse IgM F(ab)'$_2$ fragment overnight, and stained with anti-B220 and anti-CD86 (B7-2) or anti-CD69 mAb. Representative of three separate experiments. (B) BLNK$^{-/-}$ B cells do not proliferate in response to BCR cross-linking. Purified B cells from wild-type and BLNK$^{-/-}$ mice were stimulated with increasing concentrations of goat anti-mouse IgM F(ab)'$_2$ fragment for 46 h and cell proliferation was quantified in a MTT colorimetric assay.

expression of CD69 or CD86, indicating that they are non-responsive to activation via BCR cross-linking.

Activated wild-type B cells also undergo cell proliferation upon BCR cross-linking in a manner proportional to the concentration of stimulating anti-IgM mAb present (Fig. 6B). However, BLNK$^{-/-}$ B cells do not proliferate even in the presence of increasing amount of stimulant given. Thus, these data indicate that BLNK$^{-/-}$ B cells are non-responsive to BCR cross-linking *in vitro*.

Impaired T cell-independent but not T cell-dependent immune responses in BLNK$^{-/-}$ mice

Antigens that elicit an antibody response from B cells can be classified either as T independent or T dependent according to their dependency on CD4$^+$ T cell help. To examine whether BLNK$^{-/-}$ mice can mount an efficient immune response to exogenous antigens, we first immunized mice with NP coupled to Ficoll (NP-Ficoll), a T cell-independent antigen. The primary antibody response to NP-Ficoll is mainly of the IgM and IgG3 class. As can be seen in Fig. 7(A), BLNK$^{-/-}$ mice showed undetectable IgM or IgG3 antibody response to NP-Ficoll 8 days after immunization compared to the wild-type control.

For the T-cell-dependent immune response, we immunized mice with NP conjugated to CG (NP-CG). As shown in Fig. 7(B) and in contrast to the situation for the T cell-independent antigen, BLNK$^{-/-}$ mice can mount an effective immune response to NP-CG that is comparable to wild-type animals, both in terms of IgM and IgG1 secretion. Thus taken together, the data indicate that BLNK$^{-/-}$ mice have an impaired immune response to T-independent but not T-dependent antigens.

Discussion

Mice deficient for the adaptor protein BLNK/SLP-65 exhibit a severe block in early B cell development at the pro-B to pre-B cell transition stage where the pre-BCR is expressed. This is consistent with the notion that a signal from the pre-BCR is required for the progression of early B lymphopoiesis (1) and presumably BLNK is needed for the transduction of such a developmental signal. However, this early developmental block is incomplete as sIgM$^+$ B cells do develop that could eventually seed the peripheral lymphoid tissues. It is not known currently why and how these sIgM$^+$ B cells could bypass the developmental block at the pro-B to pre-B cell transition stage. It is intriguing to speculate that perhaps these sIgM$^+$ B cells encode for polyreactive antibodies that recognize certain environmental or self ligands with higher affinities and this heightened interaction provides the signal that could compensate for BLNK deficiency during the developmental process. This possibility can be readily tested by breeding Ig heavy and light chain transgenic mice bearing polyreactive or autoreactive antibodies with BLNK$^{-/-}$ mice. Such experiments are in progress in the laboratory.

Interestingly, BLNK$^{-/-}$ mice failed to generate a population of IgM^{low}IgD^{high} B cells in the periphery and lack B-1 cells in the peritoneal cavity. In addition, BLNK$^{-/-}$ mice could not mount an effective humoral immune response to T cell-independent antigens while maintaining a normal T cell-dependent immune response. While the current work is in progress, two other groups have also generated mice deficient in BLNK or SLP-65 (22,23). The phenotypes of the three independently generated BLNK mutant mice (22,23 and current study) are comparable and together confirmed the physiological role of BLNK in B cell development. However, we differ with respect to the inability of our BLNK$^{-/-}$ B cells to up-regulate the expression of the activation markers CD69 and CD86 upon anti-IgM stimulation *in vitro*. This difference could be due to the use of an enriched population of B cell in our assay as compared to the use of total splenocytes by the other groups (22,23). It is conceivable that in the latter, other factors such as the availability of T cell help in the form of secreted cytokines might overcome the inability of BLNK$^{-/-}$ B cells to respond to anti-IgM stimulation *in vitro*. Another possible explanation for the difference in our data could be the difference in the timing of assessment of the activation of BLNK$^{-/-}$ B cells. In our study, we examine the up-regulation of the activation markers after an overnight stimulation of <18 h. Since BLNK is an adaptor molecule that facilitates the interaction of other proteins, the absence of BLNK may simply lead to a slower kinetic of activation of mutant B lymphocytes compared to wild-type cells. Indeed, further experiments will have to be conducted to determine the kinetics (if any) and parameters of activation of BLNK$^{-/-}$ B cells *in vitro*. Finally, our data indicating the inability of BLNK$^{-/-}$ B cells to proliferate

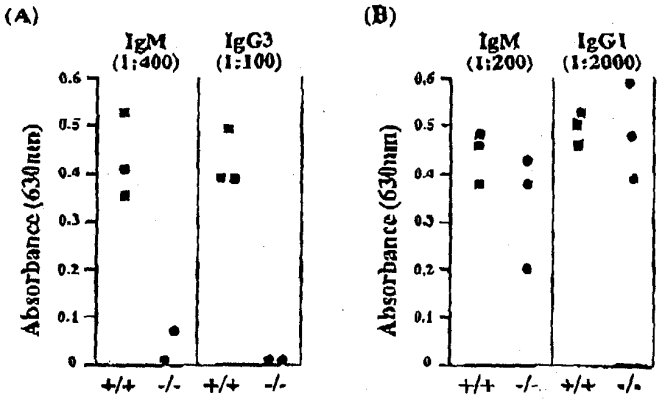

Fig. 7. BLNK$^{-/-}$ mice have impaired immune response to T cell-independent but not T cell-dependent antigens. Groups of three wild-type (■) and BLNK$^{-/-}$ mice (●) were immunized i.p. with (A) 10 μg NP-Ficoll, a T cell-independent antigen, and (B) 100 μg alum-precipitated NP-CG, a T cell-dependent antigen. Sera were collected 8 days after immunization and quantified for the presence of NP-specific antibodies of various Ig classes in an ELISA using NP-BSA as the coating antigen. The immune sera were diluted several fold and the absorbance values for the indicated dilution (e.g. 1:100 for IgG3 in the T cell-independent immune response) were plotted. Pre-immune sera were negative for the presence of NP-specific antibodies and are not shown.

and be activated by anti-IgM stimulation *in vitro* would correlate much better with the inability of these mutant B cells to mount a T cell-independent immune response *in vivo*.

It has been shown that BCR expression is required for the maintenance of peripheral B cells (5). Our additional data on the accumulation of peripheral B cells in BLNK$^{-/-}$ mice with age suggest that signals transduced by BLNK are not involved in the persistence or maintenance of lymphocytes. This is in contrast to the situation in mb-1Δc/Δc mice that have a compromised BCR and in which the peripheral B cell pool does not expand with increasing age of the animals (4). These data together would imply that the cell survival signal mediated by Igα in the BCR complex is not propagated by BLNK. Although BLNK$^{-/-}$ B cells can accumulate in the periphery, the majority of these cells failed to mature to a IgM^{low}IgD^{high} stage, suggesting that BLNK-transduced signal is needed for the final maturation of B lymphocytes in the secondary lymphoid tissues.

The developmental and functional defects in BLNK$^{-/-}$ mice bear striking resemblance to *xid* mice that lack Btk (24–26). Both mutant mice have a block in primary B lymphopoeisis, lack B-1 and mature B cells but accumulate immature IgM^{high}IgD^{low} and IgM^{high}IgD^{high} cells in the periphery; and are unable to mount immune responses to T-independent antigens. To a lesser extent, the B cell developmental defect in BLNK$^{-/-}$ mice is also similar to that of mutant mice lacking the tyrosine kinase Syk (27,28) and to mice with disruption of the p85α subunit of phosphoinostitide 3-kinase (PI-3K) (29,30). All these mutant mice had a block in the pro-B to pre-B cell transition and lack the IgM^{low}IgD^{high} peripheral B cell fraction. BLNK$^{-/-}$ mice also bear a similarity in phenotype to mice that lack the proto-oncogene Vav (31–33) in that they both have increased number of IgM^{high}IgD^{low} cells and lack B-1 cells. The similarity in the phenotypes of these various mutant mice is not surprising considering that Syk, Btk, Vav, BLNK and perhaps also PI-3K could interact with each other biochemically (7,8,10,11). BLNK has been identified as the major substrate phosphorylated by Syk that leads to calcium mobilization by PLC-γ2 (11). Recently, it has been shown that BLNK can also bind to the SH2 domain of Btk (10). Perhaps, it is this disruption in Btk–BLNK interaction that is responsible for the lack of B-1 cells in BLNK$^{-/-}$ mice. It remains to be established whether B-1 cells failed to be generated or, if generated, fail to be maintained in BLNK$^{-/-}$ mice.

Btk mutation is responsible for X-linked agammaglobulinemia (XLA), a human immunodeficiency syndrome (34). A recent report indicates that mutation in BLNK can also cause human immunodeficiency (35). Since BLNK$^{-/-}$ mice resemble *xid* mice, it is now of great interest to establish whether mutation in BLNK may be responsible for a large proportions of the human immunodeficiency that is not associated with a mutation in Btk. This is of particular significance as BLNK is expressed only in B cells and, unlike Syk (27,28) or PI-3K (29,30), its deficiency does not result in embryonic lethality.

Finally, disruption of SLP-76 in T cells leads to a complete block in T cell development (14,15), whereas IgM^{-} B cells can develop in the absence of BLNK/SLP-65 (22,23 and current study). This would suggest that although the ordered pathways for the development of T and B are quite similar, their mechanisms for the control and regulation of maturation might be quite different in some aspect (36). The availability of BLNK$^{-/-}$ mice will no doubt aid in the further study of the B cell differentiation process.

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Abbreviations

BCR	B cell receptor
BLNK	B cell linker protein
Btk	Bruton's tyrosine kinase

CG	chicken globulin
ES	embryonic stem cell
NP	4-hydroxy-3-nitrophenyl acetyl
PI-3K	phosphoinostitide 3-kinase
PLC	phospholipase C
SLP	SH2 domain-containing leukocyte protein
xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

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The B cell-restricted adaptor BASH is required for normal development and antigen receptor-mediated activation of B cells

Katsuhiko Hayashi*, Ryo Nittono*, Noriaki Okamoto*, Sachiyo Tsuji*, Yasushi Hara*, Ryo Goitsuka*†, and Daisuke Kitamura*‡

*Research Institute for Biological Sciences, Science University of Tokyo, and †Inheritance and Variation Group, Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Yamazaki 2669, Noda, Chiba 278-0022, Japan

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B cell antigen receptor signals development, activation, proliferation, or apoptosis of B cells depending on their condition, and its proper signaling is critical for activation and homeostasis of the immune system. The B cell-restricted adaptor protein BASH (also termed BLNK/SLP-65) is rapidly phosphorylated by the tyrosine kinase Syk after BCR ligation and binds to various signaling proteins. BASH structurally resembles SLP-76, which is essential for T cell development and T cell receptor signaling. To evaluate the role for BASH in B cell development and function *in vivo*, we disrupted BASH alleles in embryonic stem cells by means of homologous recombination and used these cells to complement lymphocyte-incompetent blastocysts from RAG2-deficient mice. In the resultant chimeric mice, T cell development was apparently normal, but B cell development was impaired, and a normally rare population of large preB cells expressing preB cell receptor dominated in the bone marrow in place of small preB cells, although they were mostly noncycling. In addition, the mature B cell populations in the periphery and the bone marrow profoundly decreased in size, as did B-1 cells in the peritoneal cavity, and serum Ig was severely reduced. The BASH-deficient B cells scarcely proliferated or up-regulated B7-2 in response to BCR ligation and poorly proliferated upon CD40 ligation or lipopolysaccharide stimulation. This phenotype indicates that BASH is critical for preB cell receptor signaling inducing proliferation of large preB cells and the following differentiation, for peripheral B cell maturation, and for BCR signaling inducing activation/proliferation of B cells.

Antigen receptor complex on B cells (BCR) is composed of a membrane-form of Ig H chain, Ig L chains, and Ig-α/Ig-β (CD79a/b) cytoplasmic components. Upon binding to antigen, BCR generates signals provoking various events on B cells, such as cytokinetic change to internalize the antigen, cell activation manifested as up-regulation of surface proteins including B7-2 (CD86), increase in size and cell-cycle entry, and survival or, in some condition, apoptosis. Such cellular events are prerequisites for collaboration with T cells, clonal expansion, and terminal differentiation to antibody-secreting or memory cells, or self-tolerance (1). Recently, it was shown that the continued presence of BCR, without obvious antigen binding, is required for the persistence of mature B cells (2), suggesting that BCR on the cell surface constitutively signals the survival and perhaps maturation of the cells. The preBCR has a similar structure to BCR, and is composed of Ig-α/Ig-β, μH chain (μH) and the surrogate L chains, $\lambda 5$ and V_{preB}. The formation of the preBCR is critical for early B cell development because the mice lacking the components of the preBCR exhibit a developmental block at the proB cell stage (3–8) and loss of H chain allelic exclusion (9, 10). The developmental block presumably stems from the lack of preBCR-signaled proliferation and/or survival of preB cells and the following differentiation into small, resting preB cells, the major site of L chain gene rearrangement (11, 12).

Ig-α/Ig-β transduces the signal from BCR or preBCR primarily through the activation of tyrosine kinases such as Src-family kinases, Lyn, Fyn, Blk, or Syk and Btk kinases. These kinases phosphorylate various signaling intermediates, including enzymes such as phospholipase C (PLC)-γ2, phosphatidylinositol 3-kinase, and Vav, or adaptor molecules such as Shc, Grb2, Nck, and HS1 (13–15). Deficiency of either one of the Src-family kinases did not cause serious impairment of B cell development or weakening of BCR signaling. On the other hand, Syk deficiency caused early block of B cell development at proB cell stage, indicating that Syk is primarily responsible for preBCR signaling (16, 17). Mutations in the *btk* gene were identified as being responsible for X-linked agammaglobulinemia (Bruton's disease) in humans and X-linked immunodeficiency (*Xid*) in mice. *Xid* mice, as well as *btk* gene knockout mice, are characterized by the impaired function of peripheral B cells, primarily because of deficiency of BCR signaling (ref. 18, and the references therein). Studies have evidenced that Syk and Btk are necessary for activation of PLCγ2 and the following intracellular calcium flux (19–22). However, it is unclear how these kinases interact with PLCγ2 or other substrates.

Recently, we isolated a molecule that we termed BASH (for B cell adaptor containing SH2 domain) as a molecule expressed selectively in B cells in the bursa of Fabricius in the chicken (23). BASH was structurally similar to the T cell adaptor protein SLP-76 (24), became tyrosine phosphorylated upon BCR crosslinking, and bound to Syk and Shc. We also isolated a mouse homologue of BASH whose amino acid sequence was identical to BLNK, with four amino acid differences, and to SLP-65. BLNK was purified as 70/68-kDa phosphoproteins bound to the SH2 domain of PLCγ1 (25, 26), whereas SLP-65 as a 65-kDa protein rapidly tyrosine-phosphorylated upon pervanadate treatment in the presence of surface BCR (27, 28). BASH/BLNK/SLP-65 transcripts and proteins were dominantly expressed in tissues containing B cells, although a weak expression was detected in other tissues, and restricted to B-lineage cell lines. BASH/BLNK/SLP-65 was phosphorylated primarily by Syk, translocated into the membrane fraction after BCR stimulation, and bound to PLCγ1/2, Grb2, Vav, and Nck (23, 26, 27). It has been proposed that phosphorylated BLNK recruits PLCγ1/2 to the proximity of Syk, thus facilitating tyrosine phosphorylation and activation of PLCγ1/2 by Syk, and elevation of intracellular calcium upon BCR stimulation (26). Analysis of a chicken B cell line deficient for BLNK confirmed this and further revealed that BLNK is necessary for activation of JNK and p38 upon BCR ligation (29). Recently, it has been shown that BLNK also binds to Btk and mediates the phosphor-

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Abbreviations: BCR, B cell receptor; ES, embryonic stem; PE, phycoerythrin; SA, streptavidin; HRP, horseradish peroxidase; LPS, lipopolysaccharide; PLC, phospholipase C.

‡To whom reprint requests should be addressed. E-mail: kitamura@rs.noda.sut.ac.jp.

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ylation of PLCγ1/2 by Btk (30). Together, BASH/BLNK/SLP-65 is proposed to function as a scaffold protein to focus various signaling effectors at the plasma membrane for phosphorylation by Syk and/or Btk.

To elucidate the *in vivo* function of BASH/BLNK/SLP-65 in the development and function of lymphoid system, we used gene targeting in the mouse. Because BASH/BLNK/SLP-65 gene was weakly expressed in several nonlymphoid tissues, like liver, kidney, and ovary in the mouse (ref. 26, and our unpublished results), and its transcripts were identified even in fertilized eggs (DDBJ accession no. C87337), we decided to apply the RAG2-deficient blastocyst complementation assay (31) to avoid possible embryonic lethality or deficiency in tissues other than B or T lymphoid tissues. Here, we describe B cell development and function in the chimeric mice whose lymphoid system is derived from homozygous BASH-mutant embryonic stem (ES) cells.

Materials and Methods

Targeted Disruption of BASH Genes and Generation of Chimeric Mice. cDNA containing complete mouse BASH coding sequence (DDBJ/EMBL/GenBank accession no. AB015290) was obtained by standard PCR and 5′-rapid amplification of cDNA ends method (Marathon cDNA amplification kit; CLONTECH) based on an incomplete cDNA sequence identified as a homologue of chicken BASH in GenBank (BCA, accession no. AJ222814). Using the mouse BASH cDNA as a probe, a λEMBL4 genomic library from TT2 ES cell line (Lifetech Oriental, Tokyo) was screened, and a 17-kb genomic fragment containing an exon encoding amino acids 39–55 of BASH and the flanking introns was isolated. A *Hin*dIII–*Xba*I genomic fragment was used in the gene-targeting vector (pBASHneotk), in which a part of the exon and the following intron (*Eco*O109I–*Eco*RI) was replaced with two fragments: (*i*) *Bam*HI–*Mlu*I fragment of pEGFP-C1 (CLONTECH) containing three-frame stop codons and an SV40 polyadenylation signal; (*ii*) Pgk-neo^r flanked with loxP sites derived from pKSTKNeoLoxP (a gift of W. Reith, University of Geneva Medical School; see Fig. 1*a*). The vector also contains HSV-*tk* gene for negative selection (32). The linearized vector was electroporated into B6III ES cells (33) and the cells were selected by G418 (0.2 mg/ml; Wako Biochemicals, Osaka) and Gancyclovir (0.5 μg/ml; Syntex, Palo Alto, CA). Drug-resistant colonies were screened for homologous recombination events by PCR with 5′-primer (5′-TGCTAAAGCGCATGCTCCAGACTG-3′) and 3′-primer (5′-ATGCTTGACAGTGTGGGCTTCTGT-3′) as described (3). Positive colonies were propagated and verified by Southern blot analysis for a precise targeted event (Fig. 1 *b*, *e*, and *f*). The heterozygously targeted clones were transfected with a vector expressing Cre recombinase and puromycin-resistant gene (Cre-Pac; ref. 34) and selected with puromycin (0.5 μg/ml) for 54 hr. The resistant colonies were tested by neomycin-sensitivity and PCR (Fig. 1*c*, and data not shown) for the deletion of Pgk-neo sequence by loxP-mediated recombination from the targeted allele. An ES cell clone that had undergone this recombination (BASH$^{+/-}$) was transfected again with pBASHneotk and screened as above to derive a homozygously targeted (BASH$^{-/-}$) clone (Fig. 1*d*). Southern blot analysis was performed with *Bam*HI-digested genomic DNA from ES-cell clones and probes A and B (Fig. 1 *e* and *f*). BASH$^{+/-}$ and BASH$^{-/-}$ ES cell clones were injected into blastocysts obtained from RAG2$^{-/-}$ mice that had been backcrossed to BALB/c mouse strain (a gift of Y. Shinkai, Institute for Virus Research, Kyoto University), as described (31).

Flow Cytometry. Single-cell suspensions were prepared from lymphoid organs, and red blood cells were lysed in hypotonic buffer. After washing, cells were stained with anti-mouse antibodies conjugated with FITC, phycoerythrin (PE), or biotin, followed by streptavidin (SA)-RED670 (GIBCO) in the latter case, and analyzed on FACSort with CELLQUEST software (Becton Dickinson).

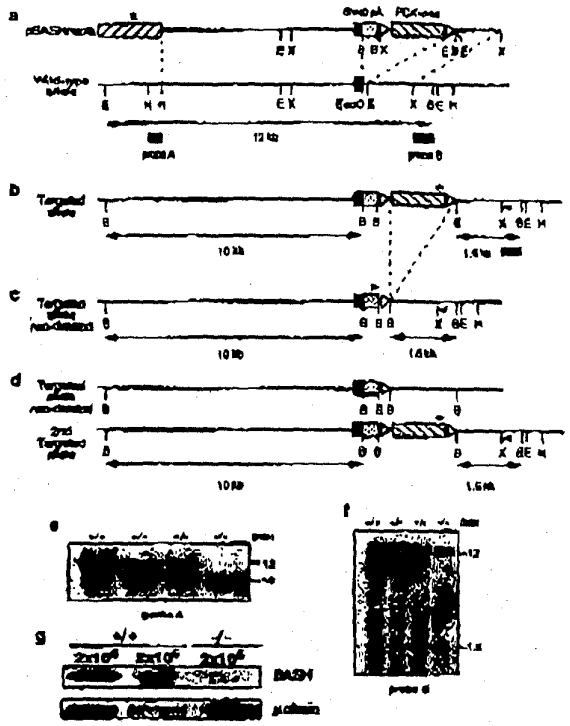

Fig. 1. Targeted disruption of the mouse BASH gene. (*a*) Structure of the targeting vector and a partial restriction map of BASH gene locus. Filled rectangles represent exons; open triangles represent *loxP* sites. Probes A and B used in Southern blot analysis are shown as boxes below a double-headed arrow, indicating a restriction fragment detected by these probes. tk, HSV-*tk* gene; pA, polyadenylation signal; E, *Eco*RI; X, *Xba*I; B, *Bam*HI; H, *Hin*dIII; EcoO, *Eco*O 109I. (*b*) Primary targeted allele. Filled triangles represent the locations of PCR primers used for the detection of homologous recombination events. (*c*) The primary targeted allele after Cre-mediated recombination of *loxP* sites, resulted in the deletion of PGK-neo cassette. (*d*) The primary and secondary targeted alleles. (*e* and *f*) Southern blot analysis of B6III cells (+/+), the cells with the primary targeted allele as in *b* (+/−, *Left*), the cells with the primary targeted allele undergone Cre-mediated recombination as in *c* (+/−, *Right*), and the cells with both targeted alleles as in *d* (−/−). The sizes of the *Bam*HI-restriction fragments hybridized with the probe A (*e*) and probe B (*f*) are indicated. (*g*) Lysates of spleen cells containing indicated number of B cells from wild-type C57BL/6 mice (+/+) or BASH$^{-/-}$RAG$^{-/-}$ chimera (−/−) were analyzed by Western blotting with rabbit anti-BASH antibody, and reprobed with anti-μH chain antibody.

Monoclonal antibodies (mAbs), FITC-conjugated anti-CD3ε (145-2C11), CD4 (RM4–5), CD43 (S7), CD25 (7D4), CD5 (53-7.3), Mac-1 (M1/70), PE-conjugated anti-CD8α (53-6.7), B220/CD45R (RA3–6B2), and biotin-anti-Ly9.1 (30C7) were purchased from PharMingen; PE-anti-IgD mAb (SBA-1), FITC-goat-anti-κ and λ, and FITC- or PE-goat-anti-IgM (μH chain-specific) were from Southern Biotechnology Associates. Biotin-goat-anti-IgM (μH) was from Cappel. Biotin-SL156 (35) was provided by H. Karasuyama (The Tokyo Metropolitan Institute of Medical Science). For the analysis of B cell activation, spleen cells were depleted of T cells as described below, stimulated with 10 μg/ml goat anti-mouse IgM F(ab′)$_2$ fragment (Jackson Laboratory) for 24 hr, then stained with PE-anti-B220 and FITC-anti-B7-2 (GL-1; a gift of R. Abe, Research Institute for Biological Sciences, Science University of Tokyo, Chiba). B220$^+$ cells were electrically gated for the analysis of B7-2 expression.

Cell Cycle Analysis. Cell sorting was performed by FACSvantage (Becton Dickinson). The sorted cells were fixed, treated with

RNase A, stained with propidium iodide, and analyzed for DNA content by FACSort, as described (36).

Proliferation Assay. B cells were enriched as follows: spleen cells were incubated on ice for 30 min in RPMI 1640 medium supplemented with 1% FCS, 25% culture supernatant of hybridoma T-24 (anti-Thy1 mAb), and 2.5 μg/ml biotin-anti-Ly9.1. After washing, T cells and Ly9.1$^+$ cells were killed with rabbit complement (Cedarlane Laboratories) and further depleted with a mixture of anti-Thy1- and SA-coated magnetic beads (Dynal, Great Neck, NY), according to the supplier's instruction. Despite many trials, enrichment of B cells from the spleen of BASH$^{-/-}$RAG2$^{-/-}$ chimera was inefficient (up to 15%) because of low quantity of B cells, compared with those of wild-type or BASH$^{-/-}$RAG2$^{-/-}$ chimeric mice (>80%). Therefore, the enriched B cells from the latter were mixed with RAG2$^{-/-}$ spleen cells, treated as above, to reproduce the proportion of B cells in the samples from BASH$^{-/-}$RAG2$^{-/-}$ chimera. In another assay, B220$^+$ cells were sorted from spleen by FACSvantage. Thus prepared samples, each containing 1×10^4 B cells, were stimulated in 100 μl culture medium containing 10 μg/ml goat anti-mouse IgM F(ab')$_2$ fragment (The Jackson Laboratory), 10 μg/ml LPS (Sigma), 10 μg/ml anti-mouse-CD40 mAb (LB429; ref. 37), or 2 ng/ml recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ), alone or in combinations, as indicated in Fig. 4. Forty hours later, [^{3}H]thymidine (0.5 μCi per well; Amersham Pharmacia) was added. Cells were harvested 8 hr later, and incorporated [^{3}H]thymidine was counted in a BetaPlate scintillation counter (Wallac, Gaithersburg, MD).

Serum Ig Titration. Igs in serial dilutions of serum were measured by ELISA using antibody pairs specific for different mouse Ig isotypes (PharMingen). 2,2'-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) was used as a substrate for the horseradish peroxidase (HRP)-conjugated secondary antibodies, and the absorbance at 405 nm was measured in a microplate reader (Bio-Rad).

Western Blot Analysis. Polyclonal anti-BASH antibody was produced by immunizing a rabbit with keyhole limpet hemocyanin-conjugated peptide corresponding to mouse BASH (amino acids 42–52). Whole-cell lysates were subjected to SDS/PAGE and blotted onto nitrocellulose filter. BASH protein was detected by the above antibody and HRP-conjugated goat-anti-rabbit or IgG (Zymed), and developed with ECL system (Amersham Pharmacia). The filter was stripped and reprobed with biotin-goat anti-mouse IgM (Cappel) and SA-HRP (Calbiochem).

Results and Discussion

Generation of Chimeric Mice with BASH-Deficient Lymphoid System. The genes encoding BASH were disrupted sequentially in C57BL/6-derived ES cells (33) by insertion of translational stop codons and polyadenylation signal into an exon coding the N-terminal portion of BASH protein by means of homologous recombination (Fig. 1 *a–d*). The BASH alleles were precisely targeted as shown by Southern blot analysis (Fig. 1 *e* and *f*). Heterozygous (+/−) or homozygous (−/−) mutant ES cell clones were injected into blastocysts from lymphocyte-deficient RAG2$^{-/-}$ mice on the BALB/c genetic background, to generate chimeric mice in which any lymphocyte must be derived from the mutant ES cells (31). Indeed, analysis of the Ly9.1 allelic marker indicated that T and B lymphocytes in the chimeric offspring were all derived from ES cells (Ly9.1$^-$), not from the recipient (Ly9.1$^+$) (data not shown). No BASH protein was detected by Western blot analysis in B cells derived from the homozygous mutant ES cells (Fig. 1*g*). Eight- to 16-week-old chimeric mice were used for the analyses shown hereafter, unless otherwise noted.

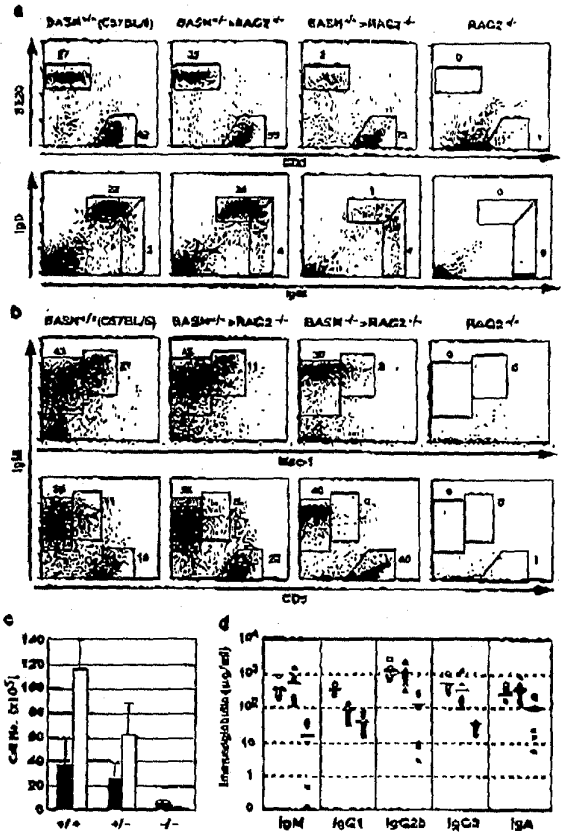

Fig. 2. Peripheral B cell deficiency in BASH$^{-/-}$RAG2$^{-/-}$ chimera. Representative flow cytometric analyses of cells from spleens (*a*) or peritoneal cavities (*b*) of the indicated mice. Cell surface antigens stained by antibodies are indicated. The number at each window indicates the percentage of the total cells within the lymphocyte gate. Spleen cells were simultaneously stained for Ly9.1 antigen, and Ly9.1$^-$ cells (derived from ES cells) were plotted and counted for the population analyses for the chimeras. Peritoneal cells were not gated by Ly9.1 marker because of its highly nonspecific staining. (*c*) Actual numbers (mean ± SD) of immature (IgM^{high}, IgD^{low}; filled bars) and mature (IgM^{low}, IgD^{high}; open bars) B cells (as defined in *a*) in spleens from wild-type C57BL/6 mice (+/+, $n = 3$), BASH$^{+/-}$RAG2$^{-/-}$chimeras (+/−, $n = 3$), or BASH$^{-/-}$RAG2$^{-/-}$ chimeras (−/−, $n = 4$). (*d*) Ig concentrations in the sera from wild-type C57BL/6 mice (○), BASH$^{+/-}$RAG2$^{-/-}$chimeras (△), or BASH$^{-/-}$RAG2$^{-/-}$ chimeras (●). Mice were 6–8 wk old. Mean values of each isotype are indicated by horizontal bars.

Severe Reduction of Mature B Cells in the Spleen and B-1 Cells in the Peritoneal Cavity of BASH$^{-/-}$ Chimeras. Chimeric mice derived from RAG2$^{-/-}$ blastocysts injected with BASH$^{-/-}$ ES cells (BASH$^{-/-}$RAG2$^{-/-}$ chimera) and those with BASH$^{+/-}$ ES cells (BASH$^{+/-}$RAG2$^{-/-}$ chimera) were analyzed for lymphocyte cellularity by flow cytometry. Population analyses shown herein were on the cells in the lymphocyte gate as defined by forward and side light scatters. C57BL/6 (B6) and RAG2$^{-/-}$ (on BALB/c background) mice were analyzed at the same time as normal and T/B lymphocyte-deficient controls, respectively. BASH$^{-/-}$RAG2$^{-/-}$, as well as BASH$^{+/-}$RAG2$^{-/-}$, chimera had normal numbers of thymocytes and no apparent defects in T cell development, as demonstrated by the expression of CD4 and CD8 on thymocytes (data not shown) and normal numbers of T cells in spleen (Fig. 2*a*). This result is consistent with the fact that BASH is not expressed in T cells (refs. 23, 26, and 27; and our unpublished results), and indicates that the generation of common lymphoid precursors from the mutant ES cells is intact.

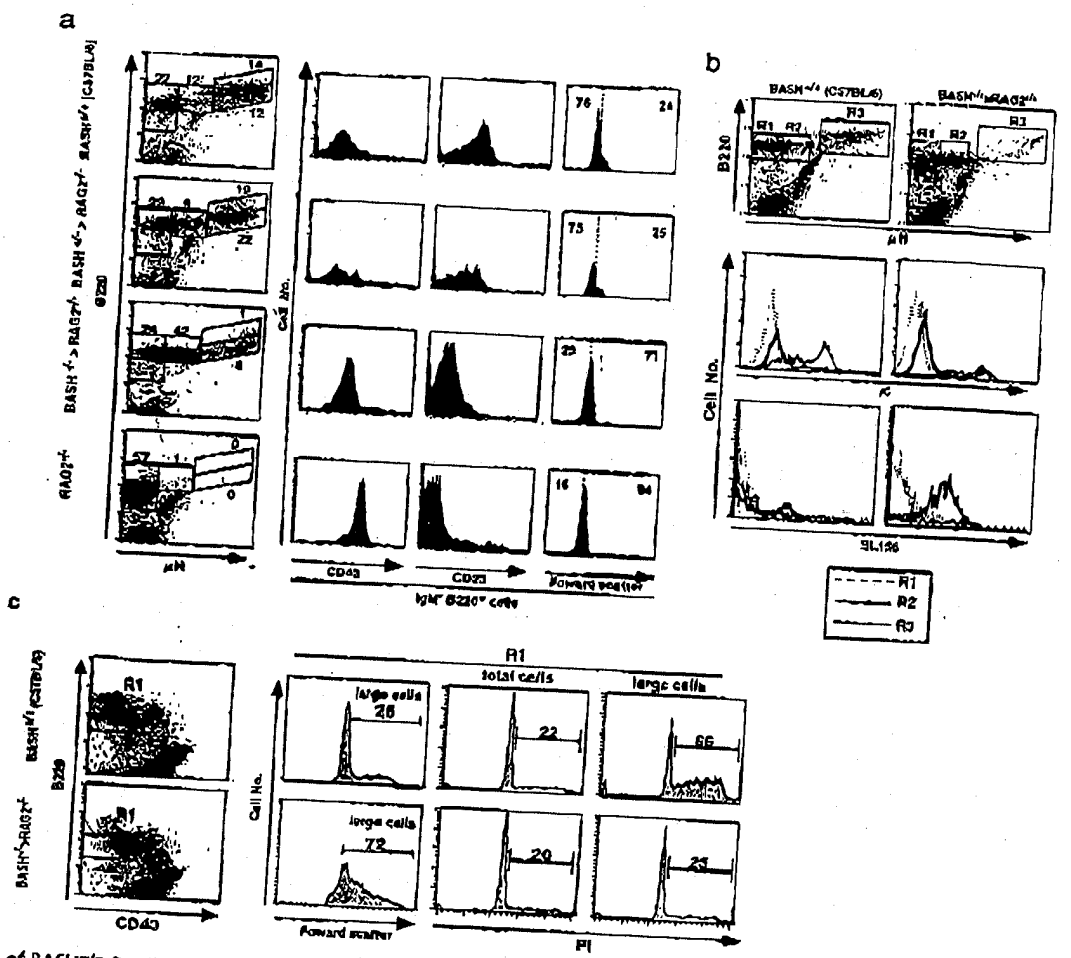

Fig. 3. Impairment of BASH$^{-/-}$ B cell development in the bone marrow. Shown are representative flow cytometric analyses of bone marrow cells from the indicated mice. Only the cells in the lymphocyte gate were analyzed. (*a*) Cells were stained for μH, B220, and Ly9.1, and Ly9.1$^-$ cells were gated for the analysis of the chimeras. B220 vs. μH expression is shown as dot plots (*Left*). The number at each window indicates the percentage of the total cells plotted. Cells were also stained simultaneously for either CD43 or CD25, and pro/preB cell fraction (the leftmost two windows set on the dot plots) were gated for the analyses of CD43 or CD25 expression, or cell size by the forward scatter. The analysis of Ly9.1 marker on proB cells (CD43^{high}, B220^{low}) indicated that, on average, about half of the proB cells in BASH$^{+/-}$RAG2$^{-/-}$ or BASH$^{-/-}$RAG2$^{-/-}$ chimeras were derived from ES cells. (*b*) Cells were stained for μH, B220, and either κL chain or preBCR (by SL156). B220 vs. μH expression are shown as dot plots (*Top*). μH$^-$, μH^{low}, and μH^{high} (IgM$^+$) cells (in the windows R1, R2, and R3, respectively) were analyzed for κL chain (*Middle*) or preBCR (SL-156; *Bottom*) expression. The nature of the bright κL chain staining on a small fraction of the μH^{low} cells from BASH$^{-/-}$RAG2$^{-/-}$ chimera (*Middle Right*, R2) remains to be elucidated. (*c*) Cells were stained for CD43, B220, and μH, then μH^{low}–μH$^-$ cells within the window (R1) set on each dot plot were sorted by flow cytometry. The sorted cells were fixed, stained with propidium iodide (PI), analyzed for cell size by forward scatter, and gated on large cells (*Left*). DNA contents of the total sorted cells (*Middle*) or the gated large cells (*Right*) were shown as histograms of PI staining, in which cells in S/G$_2$/M phases of cell cycle were indicated by bars. Numbers indicate the percentage of the total cells plotted in each histogram. The antibody-mediated fluorescence on the cell surface became negligible in the analysis of the strong PI fluorescence.

Analysis of spleen cells from the chimeras for the Ly9.1 allelic marker showed that contribution of ES cell-derived (Ly9.1$^-$) cells was relatively high (87 ± 3% in BASH$^{+/-}$RAG2$^{-/-}$ chimeras, n = 3; 73 ± 11% in BASH$^{-/-}$RAG2$^{-/-}$ chimeras, n = 4). However, B cell numbers were markedly reduced in the peripheral lymphoid organs of the BASH$^{-/-}$RAG2$^{-/-}$ chimeras. The proportion of B220 (CD45R)$^+$, IgM$^+$ B cells among Ly9.1$^-$ lymphoid cells in the spleens of BASH$^{-/-}$RAG2$^{-/-}$ chimeras was 4- to 9-fold smaller compared with BASH$^{-/-}$RAG2$^{-/-}$ chimeras or wild-type B6 mice (Fig. 2*a*). The reduction of the B cell population in the former animals was mainly the result of the disappearance of mature B cells (IgM^{low}, IgD^{high}), whereas immature (IgM^{high}, IgD^{low}) B cells were less affected (Fig. 2 *a* and *c*). This was supported by Western blot analysis showing that BASH$^{-/-}$ B cells express more μH chain than wild-type B cells on a per-cell basis (Fig. 1*g*). An almost identical phenotype was observed with lymphocytes in the blood (data not shown). These results indicate that BASH is critical for maturation and expansion of B cells in the peripheral lymphoid system.

The B cells in the peritoneal cavity consist of two phenotypically distinct subpopulations: IgM$^+$, B220^{high}, CD5$^-$, Mac-1$^-$ B-2 (conventional B) cells and IgM$^+$, B220^{low}, CD5$^+$, Mac-1$^+$ B-1 cells. The B-1 cell population was essentially absent in the peritoneal cavity of BASH$^{-/-}$RAG2$^{-/-}$ chimeras and reduced in BASH$^{+/-}$RAG2$^{-/-}$ chimeras compared with wild-type B6 mice (Fig. 2*b*), although the B-2 cell population was not reduced in BASH$^{+/-}$RAG2$^{-/-}$chimeras and only slightly in BASH$^{-/-}$RAG2$^{-/-}$ chimeras. This indicates that expansion of B-1 cells requires BASH and may depend on the dosage of BASH. Interestingly, the B-2 cells of BASH$^{-/-}$RAG2$^{-/-}$ chimeras

expressed IgM uniformly at a high level in contrast to those of wild-type mice or BASH$^{+/-}$RAG2$^{-/-}$ chimera expressing IgM heterogeneously. This, as well as the phenotype of spleen B cells, may indicate that BASH$^{-/-}$ B cells require a high amount of IgM on the cell surface to compensate for inefficient BCR signaling for expansion and/or survival (2). In accord with the reduction of B cells in the periphery, the levels of all Ig classes were markedly reduced in the sera of BASH$^{-/-}$RAG2$^{-/-}$ chimeras (Fig. 2*d*). The level of IgG1, but not the other classes, was constantly reduced also in BASH$^{+/-}$RAG2$^{-/-}$ chimeras, the reason for which is currently unknown.

Accumulation of Noncycling Large PreB Cells and the Lack of Small PreB Cells in the Bone Marrow of BASH$^{-/-}$ Chimeras. B cells are generated from hematopoietic stem cells through proB, large preB, and small preB cell stages in the bone marrow (1). As reported previously (5), B cell development was completely blocked at the proB cell stage (B220^{low}, μH^{-}, CD43^{high}, CD25^{-}) in RAG2$^{-/-}$ bone marrow cells deficient for Ig gene rearrangements (Fig. 3*a*). A similar block had been observed in mice deficient for the components of preBCR, such as membrane-form μH and λ5 (3, 4, 38); thus, it has been established that preBCR signaling is essential for the proB to preB cell transition (1). In the bone marrow of BASH$^{-/-}$RAG2$^{-/-}$ chimeras, μH^{high} (IgM^{+}), B220^{high} mature B cells were nearly absent, and the μH^{high} (IgM^{+}), B220^{low} immature B cell population was moderately reduced compared with wild-type and BASH$^{+/-}$RAG2$^{-/-}$ chimeric mice (Fig. 3*a*, *Left*). This may reflect profoundly reduced numbers of circulating B cells and inefficient generation of B cells.

Most notably, B220^{low} cells weakly expressing μH (μH^{low}) were dominant among B220^{+} BASH$^{-/-}$ bone marrow cells. Such cells were essentially absent in RAG2$^{-/-}$ mice and formed a minor population in wild-type and BASH$^{+/-}$RAG2$^{-/-}$ chimeric mice (Fig. 3*a*, *Left*). Thus, in the pro/preB cell fraction (the leftmost two windows in the dot plots in Fig. 3*a*), small preB (CD43^{-}, CD25^{high}) and large proB (CD43^{high}, CD25^{-}) cells are the majority in normal control and RAG2$^{-/-}$ mice, respectively, whereas large cells with the transitional phenotype (CD43^{low}, CD25^{low}), which correspond to the μH^{low} cells (data not shown), were the majority in BASH$^{-/-}$RAG2$^{-/-}$ chimeras (Fig. 3*a*, *Right*). Strikingly, most of the μH^{low} cells from BASH$^{-/-}$RAG2$^{-/-}$ chimeras were positively stained by the mAb specific for preBCR complex (SL156; ref. 35) but only marginally stained for κL chain (Fig. 3*b*, *Right*, R2) as well as λL chain (data not shown). The same μH^{low} cell fraction in the wild-type mice contained a few SL156^{+} cells (Fig. 3*b*, *Left*, R2) and such cells increased after culture at 37°C for 1 hour (data not shown, refs. 35, 11). Neither μH^{high} (IgM^{+}) cells nor μH^{-} cells were positive for SL156 (Fig. 3*b*, R3 and R1, respectively). These results indicate that CD43^{low}, CD25^{low}, large preB cells expressing preBCR on their surface were abnormally accumulated in the bone marrow of BASH$^{-/-}$RAG2$^{-/-}$ chimeras.

Although large in cell size, most of the BASH$^{-/-}$ large preB cells were not in cell cycle, in contrast to the normal, large preB cells (Fig. 3*c*). Therefore, BASH appears to be necessary for preBCR signaling that promotes preB cells to enter into cell cycle, and this cycling is likely to be a prerequisite for down-regulation of preBCR expression and the subsequent differentiation into small preB cells. These results also indicate, however, that BASH is not essential for preBCR signaling for the development of large preB cells from proB cells.

Despite the lack of small preB cells, the production of B cells was not severely reduced in the bone marrow of BASH$^{-/-}$RAG2$^{-/-}$ chimera. This may suggest an alternative pathway of B cell development that does not go through the small preB cell stage as suggested by the leaky B cell development in λ5-knockout mice (4, 39). In the latter mice, however, B cell generation is very inefficient because it depends on rare L chain gene rearrangements occurring on the proB cell stage (12, 38–40). Therefore, it is possible that the L chain gene rearrangement remains effective in the BASH$^{-/-}$ large preB cells because of impaired preBCR signaling, which normally down-regulates the expression of RAG genes in large preB cells (41). This possibility remains to be tested.

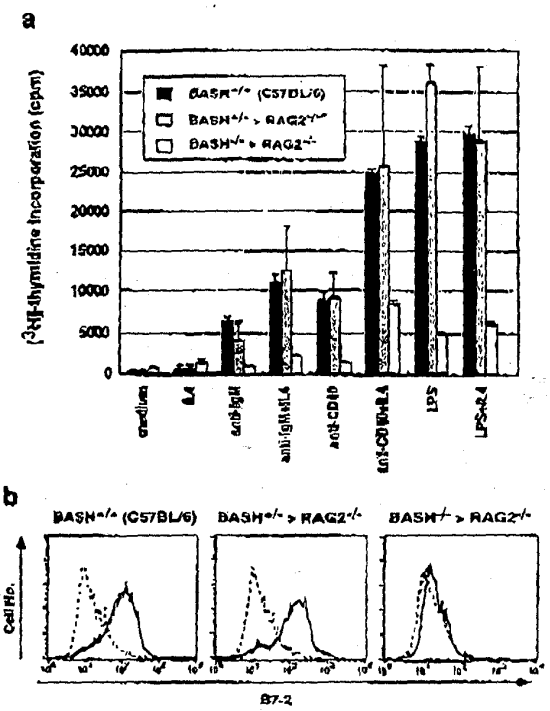

Fig. 4. Defective proliferative response and activation of BASH$^{-/-}$ B cells *in vitro*. (*a*) T cell-depleted cells from a spleen of wild-type C57BL/6 mouse, BASH$^{+/-}$RAG2$^{-/-}$ chimera, or admixed two spleens of BASH$^{-/-}$RAG2$^{-/-}$ chimeras were stimulated with the indicated reagents for 48 hr, and the incorporation of [^{3}H]thymidine was measured. Proportion of B cells in the samples was made uniform to 10%, as described in *Materials and Methods*, and that of T cells was <1%. Data are expressed as mean cpm (±SD) of duplicates. +, Separate data from two spleens of BASH$^{+/-}$RAG2$^{-/-}$ chimeras were combined. (*b*) T cell-depleted spleen cells were stimulated with 10 μg/ml goat anti-IgM for 24 hr, then stained with PE-anti-B220 and FITC-anti-B7-2 mAb (GL-1). B220^{+} cells were gated for the analysis of B7-2 expression.

BCR-Signaled Activation and Proliferation Are Impaired in BASH$^{-/-}$ B Cells. Proliferative responses to various stimuli of spleen B cells, either enriched by T cell depletion (Fig. 4*a*) or purified by cell sorting (data not shown), were examined. In contrast to the wild-type or BASH$^{+/-}$ cells, BASH$^{-/-}$ B cells negligibly responded to anti-IgM stimulation, and did poorly to anti-CD40 and lipopolysaccharide (LPS). IL-4 synergized with anti-IgM or anti-CD40 on the BASH$^{-/-}$ cells as on the wild-type or BASH$^{+/-}$ cells. BCR-mediated activation of spleen B cells was measured by up-regulation of B7-2 (CD86) antigen (Fig. 4*b*). In contrast to wild-type or BASH$^{+/-}$ B cells, B7-2 was not up-regulated on the surface of BASH$^{-/-}$ B cells after stimulation with anti-IgM antibody. These results indicate that BASH is essential for BCR-mediated activation and proliferation of B cells. Hyporesponsiveness of BASH$^{-/-}$ B cells to CD40 ligation or LPS stimulation may be the result of their immature pheno-

type, although a possibility that BASH might be involved in CD40 and LPS receptor signaling cannot be ruled out.

Although the *in vivo* immune responses have not been analyzed because they cannot be evaluated properly in chimeric mice with variable frequencies of T and B cells, so far, the observed phenotype of peripheral B cells in BASH$^{-/-}$RAG$^{-/-}$ chimeras is similar to that of Btk-knockout mice or *Xid* mice (see ref. 18 and references therein). Both are characterized by the reduced number of mature B cells (with more severe reduction in BASH$^{-/-}$RAG$^{-/-}$ chimera), the lack of the peritoneal B-1 cells, serum Ig deficiency, and defective proliferative responses to anti-IgM, anti-CD40, and LPS *in vitro*. This suggests a role for BASH in the function of Btk in the peripheral B cells. This is in line with the observations indicating that BASH/BLNK is necessary for BCR-mediated activation of PLCγ2 and the following intracellular calcium flux (29), for which Btk is also crucial (20, 21), and that BASH/BLNK binds to Btk in addition to PLC-γ2, thus mediating PLC-γ2 phosphorylation by Btk (30). Taken together, BASH may be critical for the BCR signal transduction mediated by Btk that induces maturation and activation of peripheral B cells.

During the submission of this manuscript, the phenotypes of two mutant strains of mice deficient for BASH/BLNK/SLP-65 have been published (42, 43). The phenotypes of these mutants were mostly equivalent to that of BASH$^{-/-}$ chimeric mice shown here, although the early B cell development was not analyzed in detail in the former. One marked difference is that Pappu *et al.* (43) have described that CD86 and CD69 were normally up-regulated on spleen B cells of their BLNK-deficient mice after BCR crosslinking, whereas we did not detect any significant up-regulation of CD86 (Fig. 4*b*) and CD69 (data not shown) by the same anti-IgM F(ab')$_2$ treatment. The reason for this discrepancy is unclear, but it might be because they stimulated whole spleen cells, whereas we used spleen cells depleted of T cells. The data indicating that the combination of T cell-mediated stimuli (i.e., anti-CD40 + IL-4) partially promoted the proliferation of BASH$^{-/-}$ B cells (Fig. 4*a*, and ref. 42) may support this possibility.

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BLNK Required for Coupling Syk to PLCγ2 and Rac1-JNK in B Cells

Masamichi Ishiai,* Mari Kurosaki,*
Rajita Pappu,† Katsuya Okawa,‡
Irina Ronko,† Chong Fu,† Masao Shibata,§
Akihiro Iwamatsu,‡ Andrew C. Chan,†
and Tomohiro Kurosaki*‖
*Department of Molecular Genetics
Institute for Liver Research
Kansai Medical University
Moriguchi 570-8506
Japan
†Center for Immunology
Division of Rheumatology
Departments of Internal Medicine and Pathology
Howard Hughes Medical Institute
Washington University School of Medicine
St. Louis, Missouri 63110
‡Central Laboratories for Key Technology
Kirin Brewery Company
Yokohama 236-0004
Japan
§Medical Biological Laboratories Ina Laboratory
Nagano 396-0002
Japan

Summary

Signaling through the B cell receptor (BCR) is essential for B cell function and development. Despite the key role of Syk in BCR signaling, little is known about the mechanism by which Syk transmits downstream effectors. BLNK (B cell LINKer protein), a substrate for Syk, is now shown to be essential in activating phospholipase C (PLC)γ2 and JNK. The BCR-induced PLCγ2 activation, but not the JNK activation, was restored by introduction of PLCγ2 membrane-associated form into BLNK-deficient B cells. As JNK activation requires both Rac1 and PLCγ2, our results suggest that BLNK regulates the Rac1-JNK pathway, in addition to modulating PLCγ2 localization.

Introduction

B cell receptor (BCR) engagement triggers complex cascades of biochemical events that culminate in gene transcription, cellular proliferation, and differentiation. The BCR utilizes sequential activation of at least three types of cytoplasmic protein tyrosine kinases (PTKs), Src-PTK, Syk, and Btk to regulate downstream effectors. Deficiencies of these three families of PTKs result in defective or aberrant B cell function and development (Pleiman et al., 1994; DeFranco, 1997; Kurosaki, 1997; Reth and Wienands, 1997). Thus, characterization of the substrates of these activated PTKs is a prerequisite for understanding the details of BCR-mediated signal transduction.

Two direct consequences of protein tyrosine phosphorylation have been demonstrated in the BCR signaling system. First, tyrosine phosphorylation can activate enzymes. Examples are Syk, which is phosphorylated for full kinase activity (Hutchcroft et al., 1991; Yamada et al., 1993), and phospholipase C (PLC)γ2, which upon activation generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Nishibe et al., 1990; Hempel and DeFranco, 1991; Coggeshall et al., 1992). Second, tyrosine phosphorylation creates sites for binding to proteins with SH2 domains. One example is the interaction between Igα/Igβ and Syk upon BCR stimulation. Syk SH2 domains bind to the doubly phosphorylated tyrosine residues of Igα/Igβ (Kurosaki et al., 1995; Rowley et al., 1995). In addition, tyrosine phosphorylation permits multimeric protein complex formation. For instance, Grb2 adaptor protein consists of an SH2 domain surrounded by two SH3 domains (Lowenstein et al., 1992). Grb2 SH2 domain binds to specific phosphotyrosyl residues on its target protein and the SH3 domain binds to proline-rich motifs on separate sets of target proteins such as Sos and Cbl (Saxton et al., 1994; Smit et al., 1994; Fukazawa et al., 1995; Panchamoorthy et al., 1996; Hamer and DeFranco, 1997). Thus, upon BCR stimulation, the SH2 domain of Grb2 is thought to interact with tyrosine-phosphorylated membrane proteins, thereby bringing Sos to the plasma membrane.

While much has been learned as to the relationship between the BCR-associated PTKs and downstream effectors, the molecular mechanism by which these PTKs regulate downstream events remains unclear. Analogous to receptor tyrosine kinases (Pawson and Schlessinger, 1993), it has been thought that many signaling molecules directly bind phosphorylated tyrosine residues on the cytoplasmic domains of Igα and Igβ and/or on the BCR-associated PTKs. However, this does not appear to be a feature of the coupling mechanism to downstream signaling pathways. Attention instead has focused on adaptor proteins, one of which is BLNK (alternatively named SLP-65) (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998). BLNK is a B cell–specific protein that is phosphorylated by Syk after BCR ligation. BLNK, like SLP-76 (Jackman et al., 1995), comprises a COOH-terminal SH2 domain, a central proline-rich region that binds to Grb2 SH3 domains, and multiple tyrosine phosphorylation sites.

We have investigated the mechanisms by which Syk mediates downstream effectors in B cells. Since BLNK interacts with a variety of downstream effector proteins including PLCγ2, Vav, Grb2, and Nck (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998), we hypothesized that BLNK functions as a master substrate to interface Syk with multiple downstream effectors. We now report the consequences of disruption of BLNK on the BCR-mediated responses and demonstrate a critical role for BLNK in activation of PLCγ2 and Rac1-JNK, but not Ras.

‖To whom correspondence should be addressed (e-mail: kurosaki@mxr.meshnet.or.jp).

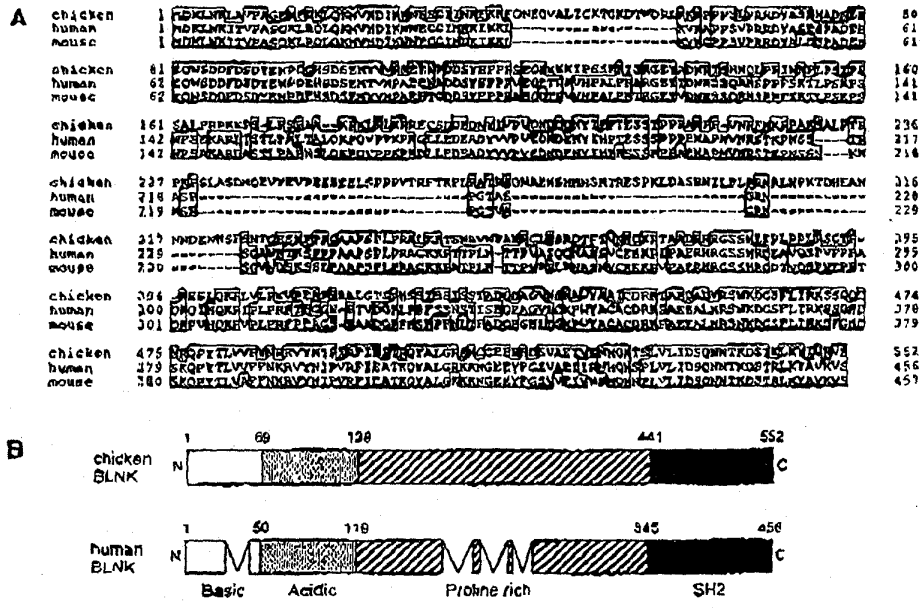

Figure 1. The Gene for Chicken pp80 Encodes BLNK

(A) Alignment of the BLNK protein. The sequences of chicken (longer form), human (longer form; pp70) (Fu et al., 1998), and mouse (Fu et al., 1998) were aligned by the Clustal W program (Thompson et al., 1994). Identical amino acids are indicated by the box. The shorter form of chicken BLNK lacks 20 amino acids (positions 36–67). Peptide sequences obtained from microsequences were as follows (amino acid number): LAVPAGEK (7–14), KPPPSLPRR (61–69), DNRTSHNQ (140–147), VPEAPSRAL (408–416), AWYAATCDRK (441–450), DGSFLIRK (462–469), QGEERFDSVAEIVEN (510–524). (B) Schematic diagrams of human and chicken BLNK. Structural domains of BLNK are shown.

Results

Chicken pp80 That Is Tyrosine Phosphorylated by BCR Ligation Is BLNK

Our previous studies have shown that BCR-evoked PLCγ2 activation is a downstream event of Syk in DT40 B cells (Takata et al., 1994). These genetic data, however, do not necessarily indicate that PLCγ2 is directly regulated by Syk in B cells. Indeed, while Syk can phosphorylate PLCγ1 in COS cells (Law et al., 1996), expression of a functional BCR, Fyn, and Syk in non-lymphoid cells does not induce PLCγ phosphorylation or increased intracellular Ca^{2+} ($[Ca^{2+}]i$) upon receptor stimulation (Richards et al., 1996). These reconstitution studies suggest the existence of B cell–specific protein(s) that may link Syk with PLCγ2 activation. Thus, we purified tyrosine-phosphorylated proteins from activated DT40 cells by affinity purification protocol based on their ability to bind an anti-phosphotyrosine MAb.

Among several purified proteins, four internal peptide sequences obtained from microsequencing of pp80 were very homologous to those of human and mouse BLNK (Figure 1A). A chicken cDNA was cloned by combination of RT-PCR method and library screening (see Experimental Procedures). Two PCR products that differed by the presence of the insertion of 20 amino acids at their N termini were detected (Figure 1 legend). Both forms contain all of the tyrosine phosphorylation sites, the proline-rich domain, and the C-terminal SH2 domain, similar to human and mouse BLNK (Figure 1B). Thus, we conclude that pp80 phosphoprotein is a chicken homolog of BLNK.

BLNK Is Required for BCR-Induced PLCγ2 Activation

To address the function of BLNK, we established DT40 B cells deficient in BLNK by gene-targeting method. Lack of BLNK expression was confirmed by Northern and Western analyses (Figures 2A and 2B). The level of cell surface expression of BCR on BLNK-deficient DT40 clone was essentially the same as that of parental DT40 cells (Figure 2C). Transcript of chicken SLP-76 in DT40 cells could not be detected by RT-PCR method (data not shown).

Comparison of the BCR-induced overall tyrosine phosphorylation between wild-type and BLNK-deficient DT40 cells did not exhibit significant changes, except that the band corresponding to BLNK itself was absent in the mutant cells (Figure 2D). These results suggest that the BCR-associated PTKs such as Lyn and Syk are activated normally in the absence of BLNK.

One of the hallmarks of the BCR-induced signaling is calcium mobilization. Fura 2-loaded wild-type and BLNK-deficient DT40 cells were stimulated with anti-BCR MAb, M4, and the rise of $[Ca^{2+}]i$ was measured. As shown in Figure 3A, no increase in $[Ca^{2+}]i$ was detected in BLNK-deficient cells. Consistent with the calcium defect, BLNK-deficient DT40 cells abolished the tyrosine phosphorylation of PLCγ2 (Figure 3C), resulting in the loss of IP_3 generation (Figure 3B) upon receptor stimulation. These defects were restored by reexpression of chicken BLNK (longer form) in the BLNK-deficient cells (Figures 2B, 3A, 3B, and 3C). These data indicate that BLNK is required for coupling Syk to PLCγ2 activation in DT40 B cells.

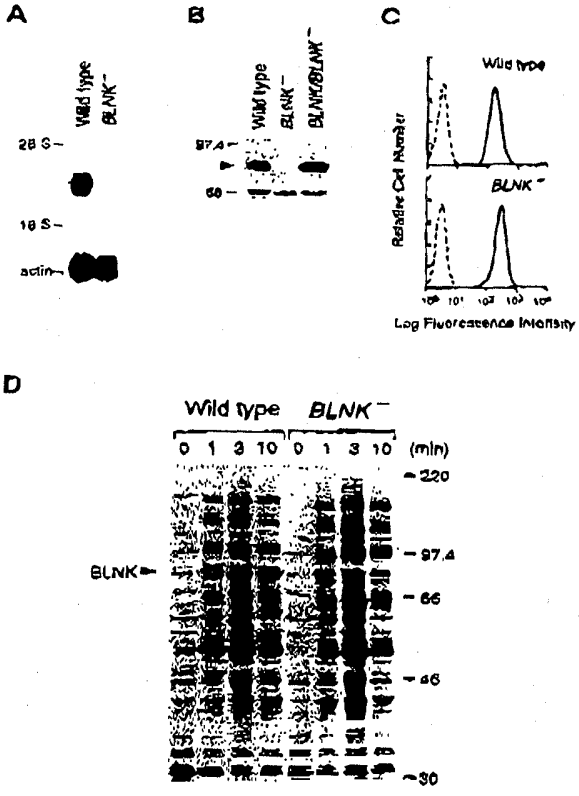

Figure 2. Disruption of the BLNK Gene in Chicken DT40 B Cells

(A) Northern blot analysis of BLNK RNA expression. RNAs prepared from wild-type and BLNK-deficient DT40 cells were separated in 1.2% formaldehyde gel, blotted, and probed with ^{32}P-labeled cDNAs chicken BLNK (top) or β-actin (bottom). The positions of the 28S and 18S rRNA are shown.

(B) Protein expression analysis of BLNK. Total cell lysate (2.5×10^6 cells) were prepared and analyzed by Western blotting using anti-BLNK Ab. Transformant of chicken BLNK cDNA (longer form) into BLNK-deficient DT40 cells is indicated as BLNK/*BLNK*⁻.

(C) Cell surface expression of BCR on wild-type and mutant cells. Unstained cells were used as negative controls (dot lines).

(D) Tyrosine phosphorylation in wild-type and BLNK-deficient DT40 cells. At the indicated time points after stimulation with M4 (4 μg/ml), whole-cell lysates prepared from 2.5×10^6 cells were loaded onto SDS-PAGE (8% gel) and analyzed by Western blotting with anti-phosphotyrosine Ab (4G10).

To formally demonstrate that BLNK acts upstream to PLCγ2 activation in the context of BCR signaling, we examined the effect of disruption of PLCγ2 on BLNK phosphorylation. Immunoprecipitation of BLNK from wild-type and PLCγ2-deficient DT40 cells demonstrated comparable levels of BLNK phosphorylation following BCR ligation (Figure 3D). Hence, tyrosine phosphorylation of BLNK by Syk likely lies upstream to that of PLCγ2.

Expression of PLCγ2 As a Membrane Chimera in BLNK-Deficient Cells Restored Calcium Mobilization

It has been previously shown that BLNK is translocated to the membrane fraction upon BCR ligation (Fu et al., 1998). Because PLCγ2 associates with BLNK in a receptor ligation-dependent fashion, we examined the translocation of PLCγ2 to the membrane fraction upon BCR aggregation in wild-type and BLNK-deficient B cells. While the stoichiometry of membrane-associated PLCγ2 was substantially enhanced following BCR ligation in wild-type DT40 cells, the translocation of PLCγ2 to the membrane fraction was markedly attenuated in BLNK-deficient cells (Figure 3E).

The above observations suggest that PLCγ2, when associated with the plasma membrane by virtue of BLNK, gains susceptibility to tyrosine phosphorylation and/or access to its substrate phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2), which in turn generates IP_3 and DAG. This hypothesis predicts that the calcium defect in BLNK-deficient cells should be suppressible by membrane expression of PLCγ2. To test this prediction, a chimera possessing the catalytic domains of PLCγ2 in place of the cytoplasmic domain of FcγRIII (mPLCγ2 shown in Figure 4A) was expressed in BLNK-deficient DT40 cells. Stimulation of the chimeric receptor alone did not induce calcium mobilization (data not shown). Although BCR ligation alone induced a small $[Ca^{2+}]i$ increase, coligation of the BCR to the FcγRIII/PLCγ2 chimera resulted in a substantial $[Ca^{2+}]i$ increase (Figure 4B). This $[Ca^{2+}]i$ increase was more sustained than that upon BCR engagement in wild-type DT40 cells (Figure 3A), suggesting that dissociation of native PLCγ2 from the membrane fraction is likely required for the proper termination of a $[Ca^{2+}]i$ increase initiated by BCR ligation.

As shown in Figure 4C, the anti-PLCγ2 Ab recognized two species of FcγRIII/PLCγ2, presumably reflecting posttranslational modifications of this molecule. As expected, coligation of the chimeric receptor and BCR induced tyrosine phosphorylation of FcγRIII/PLCγ2, the upper one of which was more prominent. Collectively, these results indicate that membrane expression of PLCγ2 is sufficient to overcome the inhibition of PLCγ2 activation observed in BLNK-deficient cells.

Ras Activation Still Occurs in the Absence of BLNK

Because the BCR-induced ERK activation is also a downstream event of Syk in DT40 B cells (Jiang et al., 1998), we examined the effect of BLNK on ERK2 activation. BLNK-deficient cells exhibited barely detectable ERK2 activation at 1 min after BCR stimulation, while this activation was observed at 3 min despite less effectively than wild-type cells. In contrast to the inhibition of the ERK2 response in the absence of BLNK, Syk-deficient DT40 cells showed the complete abrogation of the ERK2 response, as reported previously (Jiang et al., 1998) (Figure 5A).

As both PLCγ2 and Ras pathways contribute to ERK2 activation in DT40 cells (Hashimoto et al., 1998), we investigated the mechanism by which BLNK regulates ERK2. To directly determine whether BLNK affects Ras activation, we employed a binding assay developed by Taylor and Shalloway (1996). This assay is based on the observation that Raf protein has high affinity for active Ras-GTP but does not bind the inactive GDP-bound

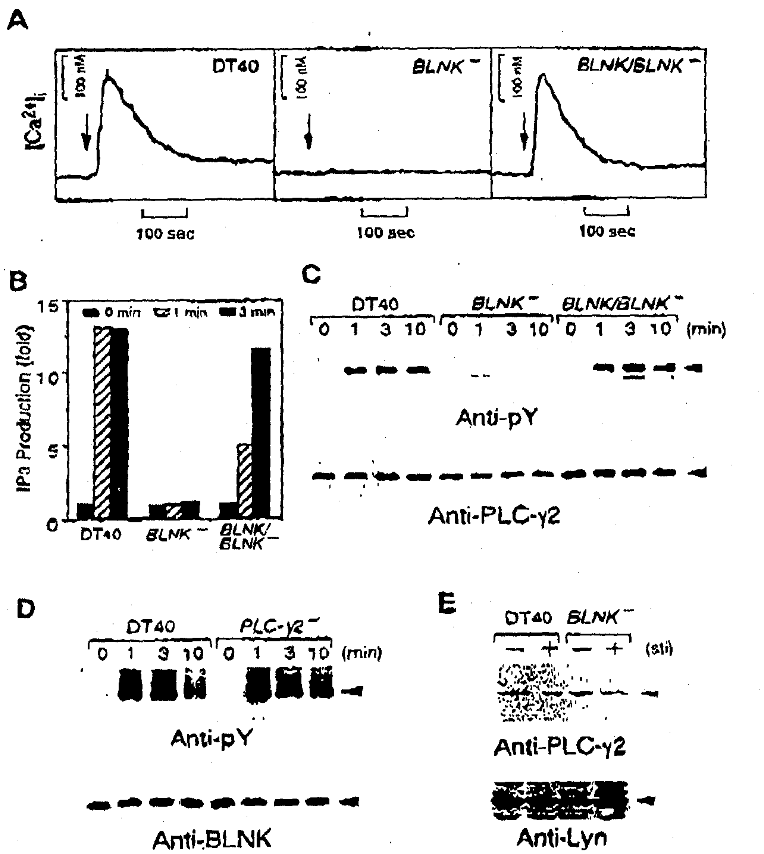

Figure 3. Characterization of BLNK-Deficient DT40 Cells

(A) Calcium mobilization analysis. Intracellular free calcium levels in Fura-2-loaded cells were monitored by a spectrophotometer after stimulation with M4 (2 μg/ml). Arrows indicate the time point for adding M4 Ab.

(B) BCR-induced IP_3 generation. Cells (2×10^7) were stimulated with MAb M4 (10 μg) for indicated time, and IP_3 productions were measured. Data are shown as fold increase of the value before stimulation with M4.

(C) Tyrosine phosphorylation of PLCγ2. At the indicated time points after M4 stimulation (4 μg/ml), immunoprecipitates with anti-PLCγ2 Ab (1×10^7 cells/lane) were separated on 7% SDS-PAGE gel and analyzed by Western blotting with 4G10 (upper). The same filter was reprobed with anti-PLCγ2 Ab (bottom).

(D) Analysis of BLNK phosphorylation in wild-type and PLCγ2-deficient DT40 cell. Cells (5×10^6 cells/lane) were similarly analyzed as in (C) using anti-BLNK Ab and 8% SDS-PAGE gel.

(E) Subcellular analysis of PLCγ2. Wild-type and BLNK-deficient DT40 cells were stimulated by M4 for 3 min (+) or left unstimulated (-). Membrane fractions were analyzed by Western blotting with anti-PLCγ2 Ab (upper) and anti-Lyn Ab (bottom).

form of Ras. BCR crosslinking of wild-type and BLNK-deficient cells both resulted in increased Ras-GTP, though the fold increase observed in the BLNK-deficient cells was somewhat lower than that in wild-type cells (Figure 5B). Hence, the significant attenuation in ERK2 activation in BLNK-deficient cells could not be accounted for by the defect of Ras activation in the mutant cells.

In order to test whether the defect in ERK2 activation by loss of BLNK was due to a defect in the PLCγ2-mediated signaling, we examined whether or not coligation of BCR and FcγRIII/PLCγ2 in BLNK-deficient cells overcomes the ERK2 inhibition. BCR-ligation alone showed a similar degree of inhibition in ERK2 activation (data not shown), while BCR-mediated ERK2 activation was restored in the mutant cells after coligation of these receptors (Figure 5A), indicating that PLCγ2 activation is sufficient to restore the ERK2 response in the absence of BLNK. Together, these data demonstrate that the attenuation in ERK2 activity observed in the BLNK-deficient cells is likely due to a defect in PLCγ2-dependent contribution to ERK2 activation. While the effect on ERK2 activation by BLNK was primarily mediated through PLCγ2, Ras undoubtedly also contributes to full ERK2 activity, as expression of a dominant-negative form of Ras (RasN17) completely abolished the residual ERK2 activity observed in BLNK-deficient cells (Figures 5A and 5C).

BLNK Is Required for the Rac1-JNK Pathway

JNK and p38 responses require Rac1, in addition to PLCγ2 activation. In fact, either expression of a dominant-negative form of Rac1 or loss of PLCγ2 almost completely abolishes the BCR-mediated activation of JNK and p38 (Hashimoto et al., 1998). Thus, to examine the role of BLNK in a Rac1-dependent pathway, we analyzed JNK and p38 responses in wild-type and BLNK-deficient DT40 cells. As shown in Figure 6, BCR-mediated activation of both JNK and p38 was completely abrogated in BLNK-deficient DT40 cells. Using a similar approach for exploring the contribution of PLCγ2 to ERK2 activation, we determined whether coligation of BCR and FcγRIII/PLCγ2 can restore the JNK and p38 responses in BLNK-deficient cells. In contrast to restoration of the ERK2 response by coligation of BCR and FcγRIII/PLCγ2 (Figure 5A), coligation of these receptors was unable to restore the BCR-mediated activation of both JNK and p38. Thus, we conclude that loss of BLNK affects not only the PLCγ2 pathway but also the Rac1-dependent pathway.

Discussion

A striking feature of BCR signaling is that a variety of distinct signaling pathways become activated by multi- ...1997).

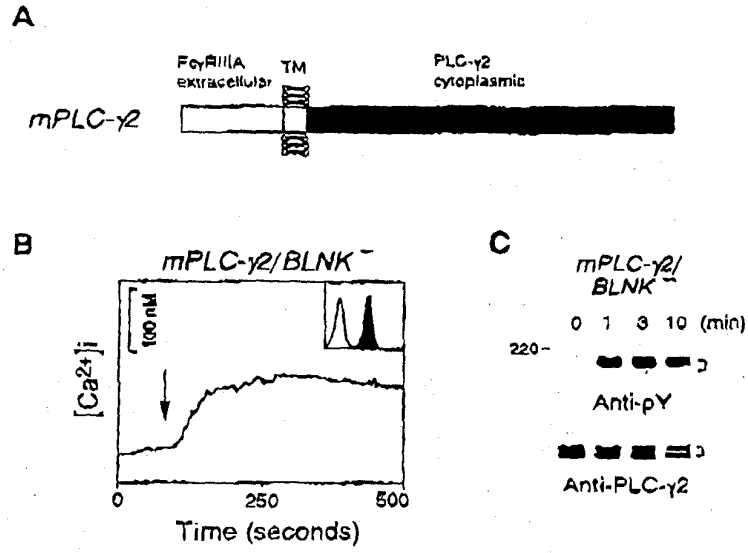

Figure 4. Expression of Membrane-Localized PLCγ2 Suppresses BLNK Inhibition

(A) Schematic diagram of membrane PLCγ2 chimera (mPLCγ2). This is composed of the extracellular domain of human FcγRIIIA (amino acids 1–212) (Ravetch and Perussia, 1989), the transmembrane domain of the human T cell receptor ζ chain (amino acids 30–58) (Weissman et al., 1988), and the complete rat PLCγ2 (Emori et al., 1989) as a cytoplasmic domain.

(B) Calcium mobilization after coligation of BCR and membrane PLCγ2 chimera. For coligation, rabbit anti-mouse IgM was added prior to stimulation with M4. Arrow indicates the addition of M4. Surface expression level of FcγRIII is indicated in an inset box.

(C) BCR-induced tyrosine phosphorylation of mPLCγ2 in BLNK-deficient DT40 cells. After coligation, immunoprecipitates with anti-PLCγ2 Ab were analyzed by Western blotting with 4G10 (upper) or anti-PLCγ2 Ab (bottom).

the key questions is the mechanism by which the BCR-activated PTKs activate the appropriate subset of signaling pathways within the cell. In the case of Syk, at least three signaling pathways (PLCγ2, Ras, and Rac1-JNK) have been identified as its downstream effectors (Takata et al., 1994; Hashimoto et al., 1998; Jiang et al., 1998). In this study, we provide genetic evidence that BLNK functions as a coupling molecule to PLCγ2 and Rac1 pathways, but not to the Ras pathway.

A model for the mechanism of BLNK-mediated activation of PLCγ2, based on the data presented here, is shown in Figure 7. Previous studies have shown that Syk is essential for tyrosine phosphorylation of both BLNK and PLCγ2 upon BCR engagement, since Syk-deficient DT40 cells fail to phosphorylate these molecules (Takata et al., 1994; Fu et al., 1998). In addition, the data shown in Figures 3C and 3D indicate that tyrosine phosphorylation of BLNK by Syk is an upstream event to PLCγ2 phosphorylation. Thus, these findings suggest that the phosphorylated BLNK brings PLCγ2 into close proximity with the activated Syk and thereby facilitates the tyrosine phosphorylation and subsequent activation of PLCγ2. Consistent with this model, a membrane chimera FcγRIII/PLCγ2 was able to overcome the defects of PLCγ2 phosphorylation and its subsequent activation observed in BLNK-deficient cells, when cross-linked to the BCR (Figure 4). The observation that the BCR-mediated translocation of PLCγ2 to the membrane fraction is inhibited by loss of BLNK (Figure 3E), together with the evidence of colocalization of BLNK with Syk (C. F. and A. C. C., unpublished data), further supports this model.

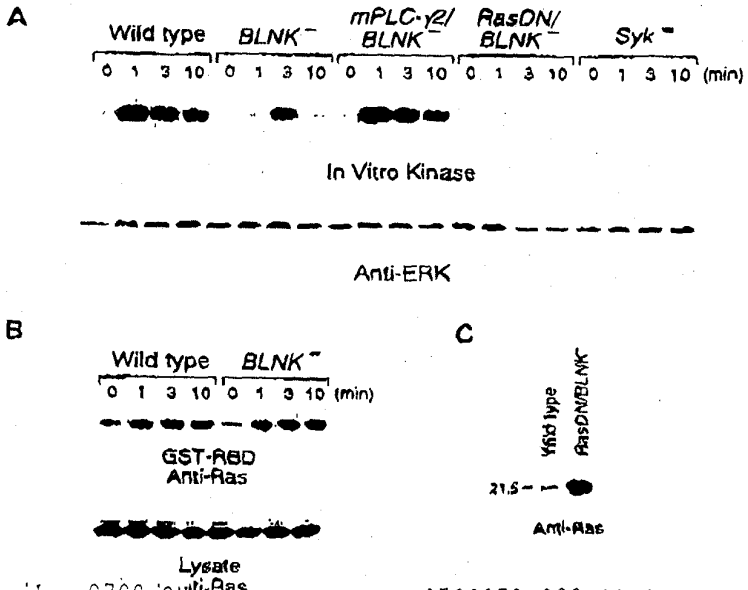

Figure 5. ERK2 Responses upon BCR Engagement

(A) Various DT40 cells were stimulated with M4 (4 μg/ml) for indicated time. For mPLCγ2/BLNK⁻, cells were incubated with anti-mouse IgM (10 μg/ml) for 5 min before M4 simulation. ERK2 was immunoprecipitated and the precipitates were assayed for kinase activity using GST-Elk1 fusion protein as a substrate. The kinase reaction products were resolved by 12.5% SDS-PAGE gel and autoradiogramed. The protein levels in immunoprecipitates by Western blot analysis were shown in a lower panel.

(B) Analysis of Ras activation by the Ras-GTP method. M4 stimulated cell lysates were subjected to affinity precipitation with GST-RBD. Activated Ras proteins were detected by Western blotting with anti-Ras Ab (upper). Similarly, the total amount of Ras protein was determined using the cell lysate (bottom).

(C) Ras protein expression in BLNK-deficient cells expressing RasN17. Expression was measured by Western blot analysis using anti-Ras Ab.

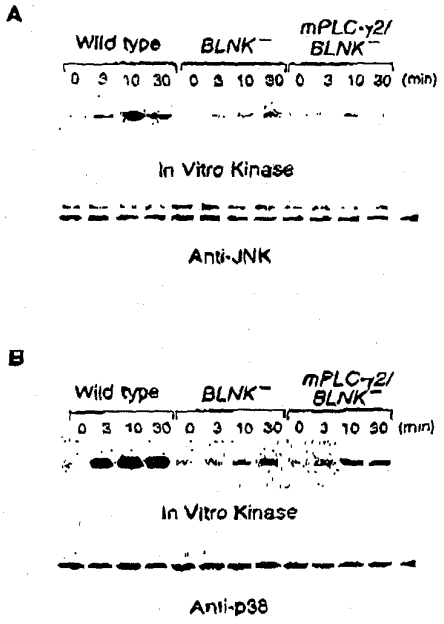

Figure 6. BCR-Induced JNK and p38 Activation in BLNK-Deficient DT40 Cells

Stimulation was carried out as in Figure 5A. After M4 (4 μg/ml) stimulation cell lysates were immunoprecipitated with anti-JNK1 (A) and anti-p38 Ab (B), kinase activities were assayed as in Figure 5A using GST-c-Jun and GST-ATF2 as substrates, respectively.

Btk, in addition to Syk, has been shown to be required for maximum tyrosine phosphophorylation of PLCγ2 upon antigen receptor engagement and its full activation (Takata and Kurosaki, 1996; Fluckiger et al., 1998). Since BCR-induced tyrosine phosphorylation of BLNK normally occurs in Btk-deficient DT40 cells (Fu et al., 1998), we propose that after PLCγ2 is recruited to the membrane fraction by BLNK, the membrane-associated Btk is then able to phosphorylate tyrosine residues on PLCγ2, leading to its full activation. Since Btk SH2 domain, in addition to its PH domain, is required for full PLCγ2 phosphorylation in B cells (Takata and Kurosaki, 1996), Btk SH2 domain might be recruited to tyrosine-phosphorylated BLNK, allowing Btk to phosphorylate PLCγ2.

As BLNK is structurally related to SLP-76, BLNK likely plays a functionally similar role in B cells as SLP-76 plays in T cells. The calcium defect observed in the SLP-76-deficient Jurkat T cell line (Yablonski et al., 1998), however, is not so complete as the phenotype observed in the BLNK-deficient DT40 B cells. This difference may be due to the residual expression of SLP-76 in the mutant Jurkat cell line, as mentioned in their report. Another possibility is that LAT, which is expressed in T but not in B cells (Weber et al., 1998; Zhang et al., 1998), cooperates with SLP-76 in regulating the calcium signaling pathway. Thus, in the case of T cells, the existence of LAT may rescue the calcium defect to some extent even in the absence of SLP-76. Additional studies are underway to further define the functional parallels between the use of these adaptor proteins in T and B cells.

We have previously shown that the synergistic action of Ras and PLCγ2 pathways is required for BCR-induced ERK2 response in DT40 cells. The ERK2 response is partially blocked by loss of PLCγ2 or expression of RasN17, while this response is completely abrogated by introduction of RasN17 into the PLCγ2-deficient mutant (Hashimoto et al., 1998). Thus, the partial inhibition of BCR-mediated ERK2 response in BLNK-deficient DT40 cells can be accounted for by the defect of PLCγ2 pathway in this mutant cell. This explanation is supported by (1) Ras activation even in the absence of BLNK, assessed by binding to GST-Raf; (2) complete inhibition of the ERK2 response by the introduction of RasN17 into the mutant cells; and (3) restoration of the ERK2

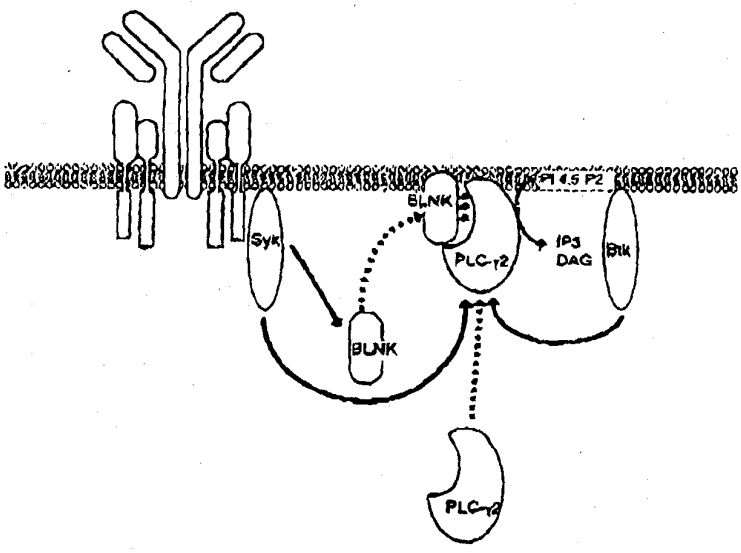

Figure 7. Model for the Mechanism of BLNK-Mediated PLCγ2 Activation upon BCR Stimulation

Upon BCR engagement, activated Syk phosphorylates BLNK, leading to its translocation to the membrane. Phosphorylated BLNK brings PLCγ2 into the close proximity with the activated Syk in the membrane, thereby facilitating tyrosine phosphorylation of PLCγ2 by Syk. Membrane-localized Btk, presumably due to interaction between its PH domain and PI 3,4,5-P_3 (Salim et al., 1996; Rameh et al., 1997; Bolland et al., 1998; Fluckiger et al., 1998), further phosphorylates other tyrosine residues on PLCγ2, resulting in its full activation. Activated PLCγ2 hydrolyzes PI 4,5-P_2, leading to the generation of second messengers, IP_3 and DAG.

response by coligation of BCR and FcγRIII/PLCγ2 chimera in the mutant cells. Given the evidence that translocation of Grb2 from the cytoplasmic to the membrane fraction is critical for Ras activation in B cells (Hashimoto et al., 1998), our data suggest that BLNK is not necessarily required for recruitment of Grb2 to the membrane fraction. Instead, Grb2 might be recruited to another linking molecule, tyrosine phosphorylation of which is mediated presumably by Syk, since BCR-induced ERK2 response is completely abrogated in Syk-deficient DT40 cells (Jiang et al., 1998) (Figure 5).

BLNK-deficient cells were unable to activate JNK or p38 following BCR ligation. Moreover, coligation of the BCR and the FcγRIII/PLCγ2, while sufficient to restore calcium and ERK responses, failed to restore JNK and p38 activation (Figure 6). Together, these results strongly suggest that Rac1 activation is defective in BLNK-deficient DT40 cells. Indeed, expression of a dominant-negative form of Rac1 in DT40 cells abrogates the JNK and p38 responses (Hashimoto et al., 1998). Previous studies have shown that Vav and SLP-76 cooperate in augmenting TCR-mediated IL-2 transcription (Wu et al., 1996; Raab et al., 1997) and that BLNK associates with Vav in activated B cells (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998). Thus, it is most likely that BLNK is required for Vav activity in B cells. Because tyrosine-phosphorylated Vav is able to catalyze GDP/GTP exchange activity on Rac1 (Crespo et al., 1997), one potential mechanism is that BLNK, like its role in PLCγ2 activation, brings Vav to the proximity of activated Syk, thereby allowing Vav to become phosphorylated.

The present findings demonstrate that BLNK functions as a switchboard, allowing PLCγ2 and Rac1-JNK pathways to be turned on. Further selection for PLCγ2 or Rac1 might be determined by phosphorylation of distinct tyrosine residues within BLNK, because BLNK has multiple tyrosine phosphorylation sites. Given that the fate of B cells depends on which set of signaling pathways is connected from the BCR under a given circumstance (Healy and Goodnow, 1998), it is reasonable to anticipate that selection of signaling pathways by BLNK is one of the mechanisms for qualitative regulation of BCR signaling, leading to appropriate biological responses.

Experimental Procedures

Cells and Antibodies

Wild-type and its derivative mutant DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. Anti-BLNK Ab or anti-PLCγ2 Ab was obtained by immunizing rabbits with bacterially expressed GST fusion protein containing chicken BLNK (79–201 amino acid region), or chicken PLCγ2 that is corresponding to rat PLCγ2 818–919 amino acid region (Emori et al., 1989), respectively. The anti-chicken IgM Ab, M4 (μ, κ) (Chen et al., 1982), which was used for stimulation of BCR, anti-phosphotyrosine Ab (4G10), anti-chicken Lyn Ab, rabbit anti-mouse IgM Ab, anti-ERK2 Ab, anti-p38 Ab, and anti-JNK1 Ab were described previously (Takata et al., 1994; Ono et al., 1997; Hashimoto et al., 1998). Anti-Ras Ab and FITC-conjugated anti-human FcγRIII Ab were purchased from Transduction Laboratories and from Pharmingen, respectively.

Expression Constructs and Transfection

Human H-Ras and chicken BLNK (longer form) cDNAs were cloned into expression vector pApuro (Takata et al., 1994). The membrane PLCγ2 chimera cDNA (mPLCγ2 in Figure 4A) was constructed by using PCR method and cloned into pApuro. The cDNA of RasN17 in pApuro vector was already described (Hashimoto et al., 1998). These cDNAs were transfected by electroporation at 550 V, 25 μF, and selected in the presence of 0.5 μg/ml puromycin (Sigma). Expression of transfected cDNA was confirmed by Western blot analysis or FACS analysis (mPLCγ2). Cell surface expression of BCR and FcγRIII were analyzed by FACScan (Becton Dickinson) using FITC-conjugated anti-chicken IgM or FITC-conjugated anti-human FcγRIII Ab, respectively. The x and y axes for the histograms indicate fluorescence intensity (4-decade-log scales) and relative cell number, respectively.

Purification of Chicken pp80

DT40 cells (1.5×10^{10}) were stimulated for 3 min at 2×10^7 cells/ml in RPMI 1640 with M4 (3 μg/ml) at 37°C. Cells were solubilized in 20 ml NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris [pH 7.5], and 1 mM EDTA) containing 50 mM NaF, 10 μM sodium molybdate, 0.1 mM sodium vanadate, and 0.1 mM pervanadate supplemented with protease inhibitors as described previously (Takata et al., 1994). After centrifugation at 13,000 × g, supernatant was precleaned with 5 ml Protein G-Sepharose (Pharmacia) and then loaded onto a 1 ml of 4G10-Protein G-Sepharose column, which was washed sequentially with lysis buffer and PBS buffer both containing 0.1 mM sodium vanadate, 0.1 mM pervanadate, and protease inhibitors. Phosphotyrosine-containing proteins were eluted with PBS containing 50 mM phenylphosphate, 0.1 mM sodium vanadate, 0.1 mM pervanadate, and protease inhibitors. Eluent was passed over Sephadex G-25 column (Pharmacia) for desalting and eluted with NP-40 buffer (0.01% NP-40, 10 mM Tris [pH 7.5], and 1 mM EDTA). Proteins were concentrated, subjected to SDS-PAGE gel, transferred to PVDF membrane (Applied Biosystems), and stained with Ponceau S. The band for pp80 was excised and digested with *Achromobacter* protease I and endoproteinase Asp-N. Digested peptides were chromatographed by reverse-phase HPLC (Wako Pure Chemical), and amino acid sequencing was performed with a gas-phase sequencer (Shimadzu, Model PPSQ-23). We obtained seven peptide sequences as shown in Figure 1A legends.

Generation of BLNK-Deficient DT40 Cells

A 384 bp BLNK cDNA was cloned by RT-PCR method using RNA from DT40 B cells. This fragment was then used as a probe for screening λ ZAP DT40 cDNA library. From 1×10^5 plaques screened, twenty positive clones were identified and seven clones were further characterized. The complete sequence of the longest insert (2417 bp encoding protein of 552 amino acids that contains all seven peptide sequences) has been deposited into GenBank (Accession No. AF089727). Four genomic clones were obtained by screening λ FIXII chicken genomic library (1.3×10^6 plaques) using the same 384 bp DNA fragment as a probe. The targeting vectors, pBLNK-neo and pBLNK-hisD, were constructed by replacing the genomic fragment containing exons that correspond to chicken BLNK amino acid residues 57–213, with neo and hisD cassettes (Takata et al., 1994). These cassettes were flanked by 4.4 kb and 2.2 kb of BLNK genomic sequence on the 5′ and 3′ side, respectively. Transfectants were selected in the presence of G418 (2 mg/ml) and clones were screened by Southern blot analysis. The pBLNK-hisD was again transfected into the neo targeted clone and selected with both G418 (2 mg/ml) and histidiol (1 mg/ml). Introduction of a single copy of each targeting vector was verified by reprobing the blots with internal neo or hisD probe. DT40 cells deficient in PLCγ2 or Syk were described previously (Takata et al., 1994, 1995).

Northern Blot Analysis

RNA was prepared from wild-type and BLNK-deficient DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) was separated in 1.2% formaldehyde gel, transferred to Hybond-N⁺ membrane (Amersham), and probed with ^{32}P-labeled chicken cDNAs BLNK and β-actin (Kost et al., 1983).

Calcium Analysis

Cells (5×10^6) were suspended in PBS containing 20 mM HEPES (pH 7.2), 5 mM glucose, 0.025% BSA, and 1 mM $CaCl_2$, and loaded

with 3 μM Fura-2/AM at 37°C for 45 min. Cells were washed twice and adjusted to 10^6 cells/ml. For mPLCγ2 chimera (Figure 4B), coligation of BCR and the chimera was carried out by adding rabbit anti-mouse IgM (10 μg/ml) followed by anti-chicken IgM MAb, M4 (2 μg/ml). Continuous monitoring of fluorescence from the cell suspension was performed using Hitachi F-2000 fluorescence spectrophotometer (Hitachi) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described (Grynkiewicz et al., 1985).

IP_3 Generation Assay

Cells (2 × 10^6) were stimulated with MAb M4 (10 μg) at 37°C for indicated time. Kinetic analysis of IP_3 production was performed using BIOTRAK IP_3 assay system (Amersham) following the manufacturer's protocol.

Immunoprecipitation and Western Blot Analysis

For immunoprecipitation, cells were solubilized in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (Takata et al., 1994), and precleared lysates were sequentially incubated with proper Abs and protein A-agarose. Lysates or immunoprecipitates were separated by SDS-PAGE gels, transferred to nitrocellulose membranes, and detected by appropriate Abs and ECL system (Amersham).

In Vitro Kinase Assay

The assay conditions were described previously (Hashimoto et al., 1998). In brief, lysates from 2–5 × 10^6 cells were immunoprecipitated by 1 μg anti-ERK2 Ab, 1 μg anti-JNK1 Ab, or 1 μg anti-p38 Ab with 40 μl protein-G Sepharose (Pharmacia). Half of immune complexes were used for in vitro kinase assay and the rest for Western blotting. Immunoprecipitates were suspended in 30 μl kinase assay buffer containing [γ-^{32}P]ATP and 5 μM cold ATP. GST-Elk, GST-c-Jun, or GST-ATF2 fusion protein (5 μg each) was added as a substrate for ERK2, JNK1, or p38, respectively. After 20 min incubation at 30°C, the reaction was terminated by the addition of SDS sample buffer followed by boiling for 5 min. The samples were separated by SDS-PAGE gels, dried, and subjected to autoradiography.

Ras-GTP Assay

Bacterially expressed GST-RBD (Ras binding domain: amino acids 1–149 of human cRaf-1 fused to GST) prebounded glutathione-Sepharose beads (15 μl packed beads, 20 μg of protein) were prepared as described (Taylor and Shalloway, 1996). Human H-Ras cDNA was transfected into wild-type and BLNK-deficient DT40 cells. Wild-type and mutant cells expressing similar levels of Ras were selected and used for this assay. M4-stimulated cell lysates in Mg^{2+}-containing lysis buffer (Taylor and Shalloway, 1996) were incubated with the beads for 30 min at 4°C. Bound proteins were eluted with SDS-PAGE sample buffer and resolved on 12.5% SDS-PAGE gel and subjected to Western blotting with anti-Ras Ab.

Subcellular Analysis of PLCγ2

Subcellular fraction was performed at 4°C as described by Bolland et al. (1998) with some modifications. Wild-type or BLNK-deficient DT40 cells (1 × 10^8 each) were stimulated with M4 (10 μg/ml) for 3 min at 37°C or left unstimulated. Cells were resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris [pH 8.0], 2 mM $MgCl_2$, 1 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium molybdate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 2 mM benzamidine, 50 mM NaF, and 40 ng/ml PMSF) and Dounce homogenized. Cell lysates were centrifuged at 1,600 × g for 10 min. The resulting supernatants were centrifuged at 100,000 × g for 30 min. Precipitates were washed twice in lysis buffer and resuspended in lysis buffer containing 1% Triton X-100. These resuspensions were centrifuged at 12,000 × g for 20 min. Proteins were separated by 7% SDS-PAGE gel and analyzed by Western blotting using anti-PLCγ2 Ab.

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